# Intrinsic effect of anionic and cationic liposomes and the effect of encapsulation of CpG C and Poly (I:C) in lipid nanoparticles on osteo-immunomodulation

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# Abstract

The initial inflammatory phase has been shown crucial for initiation of the healing cascade in bone regeneration. The modulation of the local inflammatory environment has been proposed as a promising strategy for improved bone formation after surgical intervention with bone biomaterials. The induction of mild inflammation through microbial stimuli was recently found to be involved in osteo-immunomodulation. Bacterial stimuli or pathogen associated molecular patterns act through pathogen recognition receptors. It is though that isolated pathogen recognition receptor ligands could induce a similar immune response, while being more applicable in a clinical setting. Stimulation with synthetically developed nucleic acid based pattern recognition receptor ligands Poly(I:C) and CpG oligodeoxynucleotide type C were found to increase an early osteogenic marker in human mesenchymal stem cells. The receptors for these ligands are located intracellular. A suitable lipid based carrier system is used in this research for potential optimization of the biological activity of these synthetic pathogen recognition receptor ligands. With the high level of crosstalk between the immune and skeletal system it is expected that intrinsic immune modulation of the lipid carrier system can influence osteogenic capacity. The first part of this research focussed on the intrinsic osteo-immunomodulatory effects of anionic and cationic liposomes as a lipid based carrier system. Both anionic and cationic liposomes could induce a similar inflammatory response in macrophages, as well as a dose dependant NF-κB activation in RAW-Blue™ cells. However the early osteogenic marker ALP activity, was not increased for anionic or cationic liposomes in human mesenchymal stem cells.

The second part of the research focussed on the osteo-immunomodulatory effect of Poly(I:C) and CpG oligodeoxynucleotide type C encapsulated in a lipid carrier. The encapsulation of nucleic acid based drugs like Poly(I:C) and CPG oligodeoxynucleotide type C is more efficient using lipid nano particles as a lipid carrier system. Encapsulation of CPG oligodeoxynucleotide type C in the lipid nano particle showed an increase in ALP activity in human mesenchymal stem cells compared to its free form. Encapsulation of Poly (I:C) did not show a significant increase compared to its free form. All the formulations of lipid nanoparticles could induce a similar inflammatory response in human macrophages. Interestingly, the empty lipid nanoparticle could induce almost a similar response from macrophages compared to lipid nanoparticles encapsulating Poly (I:C) and CpG oligodeoxynucleotide type C. Macrophage TNF-a production was similar for all lipid nanoparticle formulations, only IL-6 and IL-10 production were slightly increased due to transfection of the encapsulated pattern recognition ligands.

# Contents

# 1. Introduction

## 1.1 Introduction

The skeletal system acts as an internal support structure to the body, as well as various other critical functions such as haematopoiesis, protection, movement and storage or release of minerals[1]. The regenerative capacity of bone plays a pivotal role in the quality of life for millions of patients suffering from bone defects. Tumour resection, aseptic necrosis, traumatic fracture or osteomyelitis are just a number of the causes for bone defects, which often require a surgical mediation[2]. The number of performed total knee arthroplasties in the United Stated is expected to increase with 673% in the next decade[3]. It is also expected that the demand for revisions will grow with a similar percentage. Thus the need for bone biomaterials is estimated to only increase in the future. Autologous bone is one option for such procedures. The main advantages are the osteoinductive, osteoconductive and osteogenic properties along side the immunocompatibility[4]. However there are some major disadvantages. The second site morbidity, dependency on host physiology, risk of infection and supply limitations are shortcomings which necessitate innovative approaches to circumvent autologous bone harvesting[5,6]. Bone biomaterials, which are more available and practical, have been developed to act as a potential autologous bone substitute in the past decade[7].

Numerous biological and non-biological biomaterials including metals, ceramics, synthetic and natural polymers, have been studied for their applicability in bone regeneration. Each has their own advantages and disadvantages[8]. In order to overcome the limitations of each material, compositions have been used to produce bone biomaterial scaffolds. The use of different biomaterials is only a single strategy in the field of bone tissue engineering. There are many different strategies which can be utilized to influence the capacity for bone regeneration of biomaterials(Figure 1).

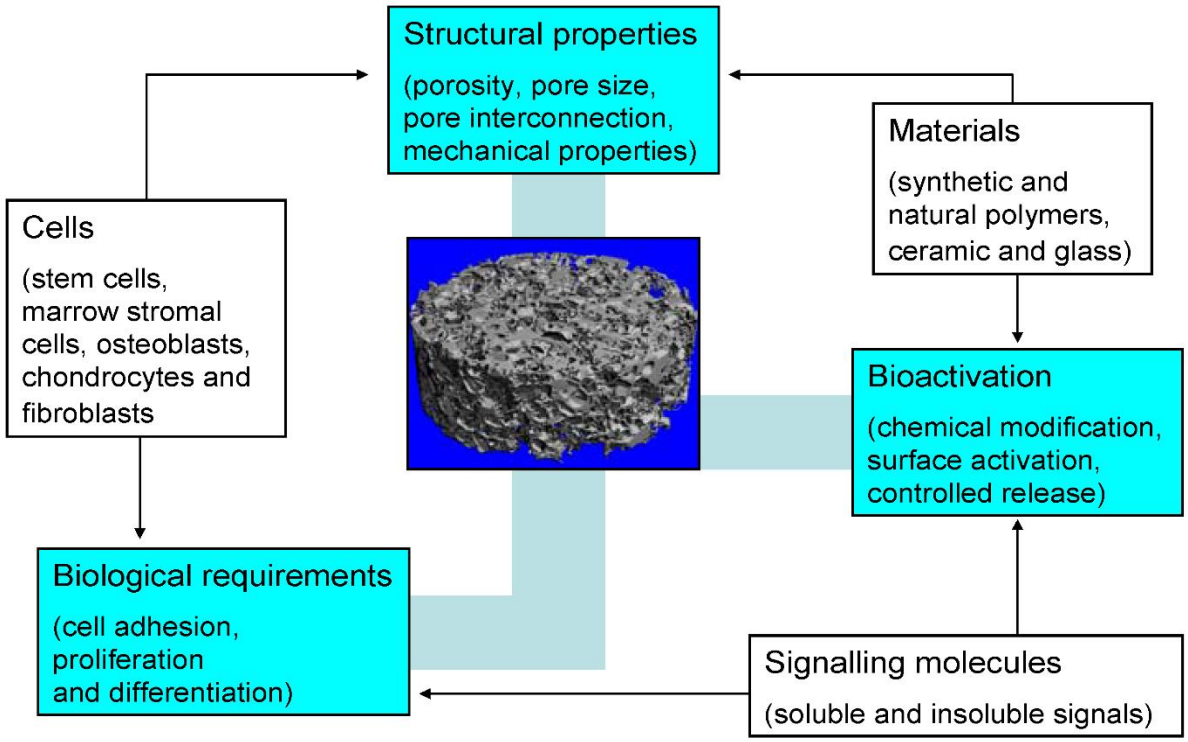


*Figure 1 - key aspects of optimizing bone biomaterial. Figure from "Bioactive Glass and Glass-Ceramic Scaffolds for Bone Tissue Engineering" L-C. Gerhardt and A.R. Boccaccini, 2010, Materials Vol 3, Pages 3867-3910. doi:10.3390/MA3073867* [9]

A challenge for bone biomaterials remains, their limited osteoinduction. Osteoinduction is the ability to create an environment primed for new bone growth, as a result of recruitment and differentiation of host osteogenic progenitor cells in conjunction with release of growth factors which promote bone formation[5,10]. In order to increase the osteoinductive efficiency of bone biomaterials, one approach is the development of a coating which aligns with the complex endogenous tissue healing process.

Modulation of local immune response has been explored as a strategy for improved bone regeneration[11]. The immune system plays a key role in the regeneration and maintenance of bone. The closely related skeletal and immune system share a variety of receptors, signalling molecules, transcription factors and cytokines[12]. The natural process of fracture healing consists off cross-talk between the skeletal and immune system. The delicate balance in the inflammatory reaction is crucial. A prolonged inflammatory state will have a negative effect on the regenerative process[13]. However the initial inflammatory phase is essential for initiating the healing cascade[14]. It has been shown that mild inflammation can be beneficial for bone formation[15]. Generally, inflammation occurs in response to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs is enabled by pattern recognition receptors (PRRs)[16]. Recent finding indicate that PAMPs could be utilized to function as an osteo-stimulatory factor[17,18]. Bacterial antigens, non-viable bacteria or their simplified analogues show potential for bone regeneration. The osteo-immunomodulation is thought to be a result of the activation of PRRs, which potentially induce and transcribe pro-osteogenic cytokines. Stimulation with synthetically developed PRR ligands Poly(I:C) and CpG oligodeoxynucleotide ( CpG C) type C were found to increase early osteogenic markers in human mesenchymal stem cells (hMSCs)[19]. The receptors for these ligands are located intracellular. A suitable transfection agent is required to optimize the biological activity for these PAMPs. A lipid based drug-carrier system is ideal for this purpose, since the morphology is similar to cellular membranes[20]. The lipid based carriers themselves elicited a immune response irrespectively of their drug load[21]. The high involvement of the immune system in bone regeneration predicts therefore a potential influence of the lipid carriers system itself. For a clinical relevant outcome, a stable coating is required for implementation PAMPs encapsulated in a lipid based carrier system. Hydrogel coatings have been developed rapidly for orthopedic applications[22]. The advantages of incorporating a lipid based carriers system inside a hydrogel are controlled release, increased stability and promotion of cell functions[23,24].

## 1.2 Research project

This research project consists of two aspects of osteo-immunomodulation by lipid based carriers. The first part focusses on the intrinsic immunomodulatory capacities of opposite charged liposomes. It consists of the synthesis of cationic and anionic lipid carries without a drug load. The osteo-immunomodulatory capacities of these liposomes will be investigated *in vitro*. The cytokine expression of macrophages and an early osteogenic marker in human MSCs will be investigated. The ability to osteo-immunomodulatory influence after incorporation of the liposomes inside a hydrogel coating for titanium implants will be analysed as well.

The second part of this projects focusses on the incorporation of PAMPs inside a lipid based carrier. Poly(I:C) and CpG ODN type C will be encapsulated inside lipid nanoparticles. The improved effect of the lipid carrier system compared to the free form PAMPs will be analysed. The osteo-immunomodulation of the lipid based carrier system encapsulating PAMP is evaluated in two stages. The *in vitro* part consists of the investigation of cytokine expression of human macrophages and osteogenic activity of human MSCs. The *in vivo* part consists of a rabbit study. The lipid nanoparticles will be applied to an implant and inserted intramuscular for analysis of bone formation.

# 2. Theoretical background

## 2.1 Bone regeneration

### 2.1.1 Bone tissue

The multifunctional bone tissue provides mechanical support, protection, haematopoiesis, mineral homeostasis and certain endocrine functions[25]. Bone tissue consists of carbonated hydroxyapatite, collagen, non collagenous proteins, water and small organic molecules[25]. The composition of the tissue, the highly organized hierarchical architecture and the continuous remodelling process of bone provide the optimal mechanical resilience and stiffness of the tissue. The bone remodelling cycle process is a vital for healthy bone homeostasis[26]. Bone remodelling is the processes of continuous bone resorption and deposition (Figure 2). The cycle is initiated by osteoclasts. The multinucleated cells are differentiated from hematopoietic stem cells through receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor 1 (M-CSF) signalling[25,27]. Osteoclasts resorb the mineralized matrix, which generates signals for the recruitment of MSCs to the site. MSCs multiply and differentiate towards osteoblasts. The osteoblasts secrete several extracellular proteins and organic matrix components like type 1 collagen, osteocalcin, alkaline phosphatase (ALP), calcium and phosphatase ions, which are needed for the mineralization of the tissue. After mineralization most osteoblast undergo apoptosis. A portion of osteoblasts become entrapped within the bone tissue, here they differentiate into osteocytes. These cells play a role in orchestrating the bone remodelling process, as well as a role as a sensory cell[28].

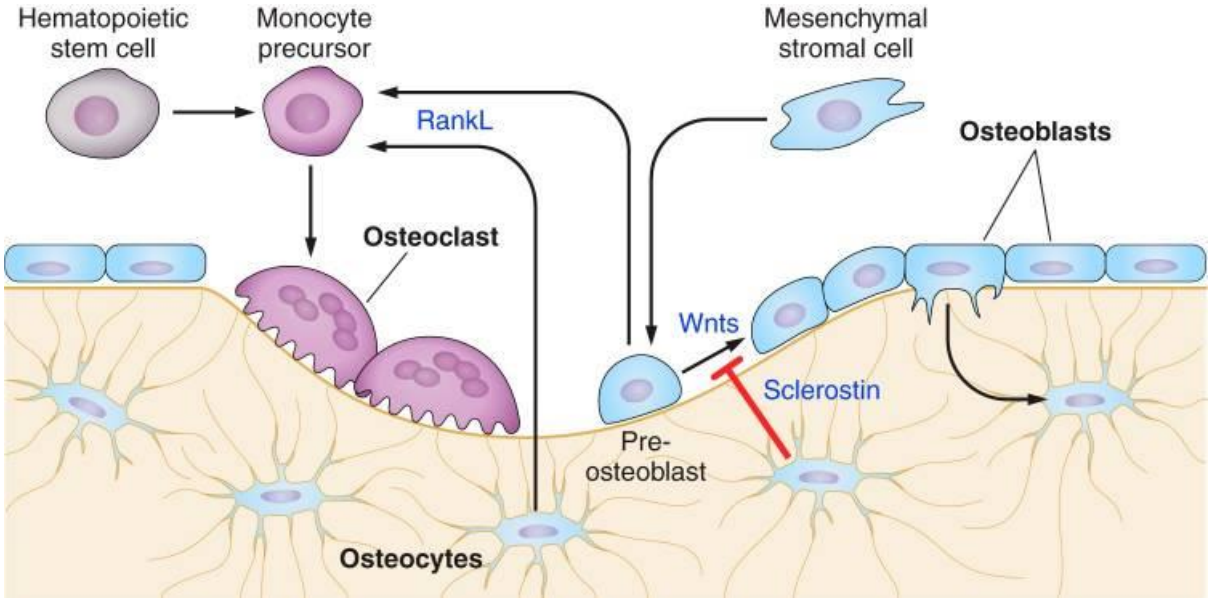


*Figure 2 – Bone remodelling and the cells involved. Hematopoietic stem cells, precursors of the monocyte macrophage lineage, differentiate into osteoclasts through signalling such as RANKL. Osteoclasts facilitate bone resorption. The bone forming osteoblasts are differentiated form MSCs and deposit new bone tissue at the excavated site. The portion of osteoblasts that become entrapped into the new bone tissue differentiate into osteocytes. Osteocytes help orchestrate the bone remodelling process through varies signals. Figure from "Bone Cell Bioenergetics and Skeletal Energy Homeostasis" Riddle, R. C. and Clemens, T. L., 2017, Physiological reviews, 97(2), 667–698 doi:10.1152/physrev.00022.2016.* [26]

The process of bone remodelling is not only vital for the maintenance bone, but also for the repair of fractures or other trauma to bone tissue[27]. The complex interaction of cells from the MSC-osteoblastic lineage and the immune cell derived monocyte-macrophage-osteoclast lineage has put the emphasis of bone homeostasis more towards the crosstalk between inflammatory cells and bone cells.

### 2.1.2 Bone regeneration and immune system

Bone tissue is unique in it's ability to regenerate itself without the formation if scar tissue[29]. In the case of a fracture the endogenous healing process is initiated. Fracture healing consists in general of two overlapping phases (Figure 3) [30]. The first phase is characterized by an increase in tissue volume, following the recruitment and differentiation of cells. A haematoma and subsequently cartilaginous callus are formed, initiating primary bone formation. In the second phase the catabolic activities become more dominant, replacing the cartilaginous callus for a harder bony callus. During this phase there is a reduction of the volume of callus tissue, bone remodelling and cartilage resorption facilitate the formation towards the bone's original structure.

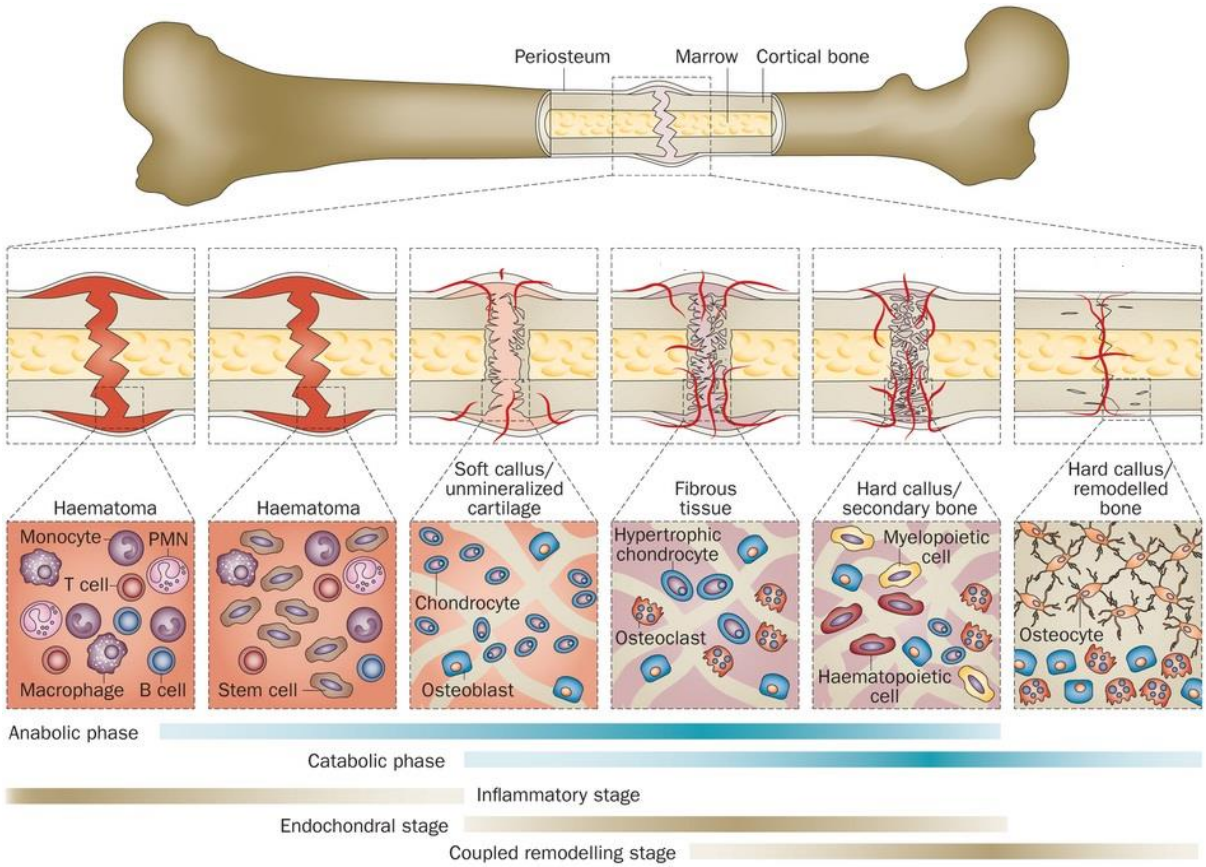


*Figure 3 - Fracture healing. The general anabolic and catabolic phases (blue bars) of bone regeneration can be seen in the context the biological stages (brown bars) of inflammation, endochondral and remodelling stage. The transformation of the Haematoma to the callus tissue into remodelled bone with the corresponding primary cells involved can be seen in framework of these phases. Adapted from "Fracture healing: mechanisms and interventions." Einhorn TA, Gerstenfeld LC., 2015, Nat Rev Rheumatol (1):45-54. doi:10.1038/nrrheum.2014.164*

The two general stages of bone regeneration can be seen in the context of three biological stages, inflammatory, endochondral and remodelling. The initial stage after injury is the inflammatory stage. The local disruption of vascularization and soft tissue will cause haematoma formation[29]. The release of DAMPs from the injured area activate the inflammatory response of the immune system. Immune cells are recruited and activated in the injured area. Removal of necrotic tissue, angiogenesis and initial repairs are initiated by cell-mediated immune functions[31].

During the inflammation phase firstly platelets arrive at the injured site and facilitate the formation of a haematoma, which can act as a scaffold for cellular engraftment[29]. Platelets release inflammatory cytokines interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α) which attract neutrophils and monocytes[32]. The released growth factors of platelets, platelet derived growth

factor (PDGF) and Transforming growth factor-β (TGF-β), recruit MSCs[33]. Neutrophils have been shown to help in the formation of the haematoma[34].However their main functions are removal of debris and secretion of cytokines[35]. The first instances after injury, there is a rapid accumulation of the short lived neutrophils. The release of IL-1, IL-6, IL-10, TNF-a, monocyte chemotactic protein-1 (MCP-1), C-X-C motif ligand 1 (CXCL1) by neutrophils attracts monocytes and helps their differentiation towards macrophages[36]. Recruited monocytes which are differentiated to macrophages can have a different polarization depending on the local environment[37]. The inflammatory M1 macrophages, which polarize under influence of pro-inflammatory cytokines IL-1 and TNF-α, perform phagocytosis and further secrete IL-1, IL-6, TNF-α and MCP-1 for the maintenance of monocyte recruitment[29]. In later stages of inflammation the M2 macrophages, under influence of anti-inflammatory cytokines such as IL-10 and Il-4, initiate an anti-inflammatory and tissue repair response. The M2 macrophages secrete tissue repair signals and recruit MSCs.

During the endochondral phase the cartilaginous callus is formed. MSCs facilitate the formation of the callus through chondrogenesis, where they differentiate into chondrocytes and form the soft callus[38]. During this phase and the overlapping remodelling phase, osteoblasts and osteoclasts are being activated. The soft callus is resorbed, MSCs differentiate into osteoblast and replace the tissue with bone tissue[31]. Monocytes differentiate into osteoclasts, which resorb the bone tissue. As the callus tissue is being resorbed, there is a continuous cycle of osteoblast and osteoclast activity. The tissue is remodelled towards the structure of the original bone.

### 2.1.3 Biomaterials in bone regeneration

Even though endogenous bone regeneration is a highly effective process, surgical mediation with an biomaterial implant is still required to replace or restore lost bone for many patients[2]. The biomaterial can function as a bone substitute in large bone defects. The material facilitates bone regeneration by interaction of the host immune cells, the host bone cells and the material. The biomaterial is a foreign material to the body, therefore a foreign body immune response is initiated[15].

In the first instance when the biomaterial comes in contact with the tissue, proteins from the blood adsorb to the surface of the material[39]. A provisional matrix is formed through the activation of blood coagulation, complement system and platelets. The signals from the provisional matrix result in the activation of neutrophils and monocytes, initiating acute inflammation. The inflammation reaction is amplified by the release of inflammatory cytokines by neutrophils and mast cells[40]. The released chemoattractant and cytokines result in the differentiation of monocytes into macrophages. In the early stage the macrophages will have a inflammatory M1 polarization, important in maintain inflammation and phagocytosis of debris. If phagocytosis is not possible due to the size of the particle, macrophages can coalesce to form foreign body giant cells (FBGC). The FBGC in collaboration with released degradative enzymes try to degrade the foreign body[2]. In the later stage the regulatory wound healing M2 macrophage polarization becomes more dominant. MSCs and monocytes, recruited during the inflammatory phase, differentiate into osteoblasts and osteoclasts. Osteoblasts deposit new bone tissue on top of the biomaterials or were the biomaterial has degraded. Osteoclasts resorb the bone tissue, initiating the cycle of bone remodelling crucial for functioning bone formation.

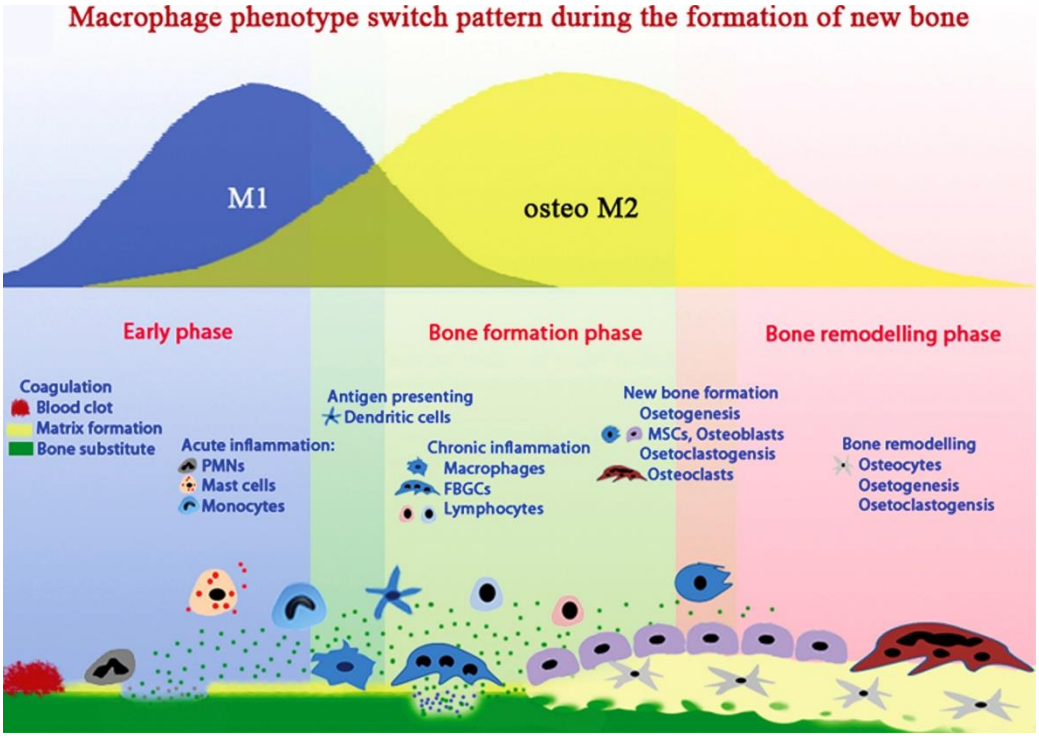


*Figure 4 – Foreign body response towards bone biomaterials. The three phases (early, bone formation and bone remodelling) can be seen with the involvement and interaction of the coagulation system, immune system and skeletal system. Adapted from "Osteoimmunomodulation for the development of advanced bone biomaterials" Chen et al., 2016, Materials Today 19 (6): 304-321, doi.org/10.1016/j.mattod.2015.11.004.*

The close relationship between the skeletal system and the immune system proposes an opportunity for biomaterials to influence the success of an implant. A favourable immune reaction to the biomaterial has the potential to generate an osteogenic microenvironment, thereby improving the induction of bone formation[2]. On the other hand, an unfavourable immune reaction could lead to implant failure[41].

Each material has its own advantages and disadvantages. Titanium scaffold for example have a high mechanical strength, however lack osteoinductive properties[8]. In contrast, Biphasic calcium phosphate (BCP) scaffolds have relative good osteoinduction and favourable degradation, but lack mechanical strength[15]. The type of material used itself has a high impact on the regenerative capacity, it determines the bulk chemistry and degradational behaviour. The rate of degradation is important for mechanical stability of the scaffold as well as the rate of released degraded products[42]. The composition of released molecules and the concentration can have various influences on the cellular behaviour, osteogenesis and angiogenesis[43]. The surface composition of a scaffold can influence the primary biological response. Topography, surface charge, wettability, porosity and pore size all have influence on the initial immune response of the body, thereby influencing the subsequent bone formation[2,41].

The surface of the material plays a crucial role in directing cellular behaviour, since the surface is the interface through which the cells initially interact with the material. Thus a common approach for optimising biomaterials is tailoring the surface composition to allow control over initial cell behaviour. Extra cellular matrix proteins, growth factors, or specific drugs have been tethered on the surface of the scaffold, allowing influence of the initial cellular behaviour[44,45]. Another approach is the release of bioactive molecules or specific drugs from the scaffold. The release can be achieved by the incorporation of the molecule into the scaffold itself, then the active molecule is released through degradation of the scaffold. The release pattern of the bioactive molecule or drug should be carefully designed for the optimal time frame in the regenerative process. The action period and dose should enable the optimal biological response. In order to improve the bioactivity of a scaffold without altering advantages mechanical or intrinsic properties, the addition of a coating to the scaffold is an option[46]. The coating can be designed for an optimal balance in stability, release and bioactivity. Furthermore, bioactive molecules ,drugs, cytokines or other proteins can be incorporated inside the coating.

### 2.1.4 Mild inflammation through PAMPs for the induction of bone regeneration

The initial inflammatory phase of bone regeneration, where proinflammatory signal recruit and differentiate immune cells, has been shown to be crucial for successful bone repair[13]. Inflammatory cytokines associated with this phase have been shown to have a pro regenerative function in the microenvironment[47]. Pro-inflammatory cytokines such as TNF-a, IL-6, IL-8, IL-17 and anti-inflammatory cytokines such as IL-10 and IL-4 are associated with the initial inflammation phase, they are though to play a role in directing progenitor cells towards the osteogenic lineage[48–52]. Modulation of the local inflammatory response has been proposed as a strategy to improve the formation of bone. The inflammatory response of the body can be triggered through various mechanisms. DAMPs, associated with damage to the tissue or cells, or PAMPs, associated with pathogens, are the main initiating signals[16].

Inducing an inflammatory response through bacterial stimuli has been shown to stimulate osteogenesis[17,18,49]. The osteogenesis is thought to be a results of the potential induction and transcription of osteogenic cytokines. Bacterial stimuli mainly act through a subtype of PRRs, Toll-like receptors (TLRs), for initiating a immune response[16]. These receptors recognize the microbial PAMPs and active the immune cells. Isolated ligands for TLRs could be more applicable in a clinical setting compared to bacterial stimuli for inducing a similar immune reaction. Pharmaceutical companies have developed synthetical adaptations of PRR ligands which resemble the microbial cell wall components or their nucleic acids[53]. Several ligands have been investigated for their capacity to direct the immune response towards an osteoinductive direction. The nucleic acid based therapeutic PRR ligands Poly(I:C) and CpG ODN type C showed the most promise for osteoinduction, they increased the early alkaline phosphatase (ALP) activity in hMSCs[19]. ALP activity can function as an early osteogenic marker[54]. The enzyme is secreted by pre-osteoblastic cell lines and takes part in calcification of the matrix. Poly(I:C) and CpG ODN both act on intracellular TLRs, Poly(I:C) is recognized by TLR 9 and CpG ODN is recognized by TLR 3[55,56]. Activation of the TLRs induces a cascade which promotes the production of inflammatory cytokines[53]. These inflammatory signals have a potential to increase the osteogenic capacities of MSCs[49].

## 2.2 Lipid-based carrier system

Nuclear acid based therapeutic PRR ligands Poly(I:C) and CpG ODN act on intracellular TLRs[55,56]. Poor membrane permeability and nuclease resistance limit the effect of nucleic acid based drugs in their free form[57]. The incorporation of these ligands inside a delivery system could enable a more effective intracellular uptake required for medical applications. Lipid based carriers systems, such as liposomes and lipid nano particles (LNPs), seem to be ideal. These particles have a morphology which is similar to the cellular membrane, allowing for intracellular uptake[20,58]. Furthermore is the charge and size tuneable for specific objectives and are several formulation already approved for clinical application. Liposomes consists of a lipid bilayer with an aqueous core (Figure 5)[59]. Hydrophilic drugs can be stored in the aqueous centre and hydrophobic drugs can be stored in the lipid bilayer. The composition of lipids, preparation method, size, charge and lipid organization all effect the suitability of the liposome as a specific carrier system. The encapsulation of nucleic acid based drugs by liposomes has been limited by low encapsulation efficiency and drug to lipid ratios[58]. Formulation of a LNP which allowed high nucleic encapsulation with stability in biological milieu has been described by Semple and colleagues[58]. Firstly the nucleic acid forms lipoplexes with the lipid, then the access lipid forms a hydrophobic core around the lipoplexes. This core is then coated by polar lipids (Figure 5)[60]. The release of the nucleic acids in the cell is based on endocytosis[61]. The acidification of the LNP lipids inside to endosome allow for fusion with the endosomal membrane.

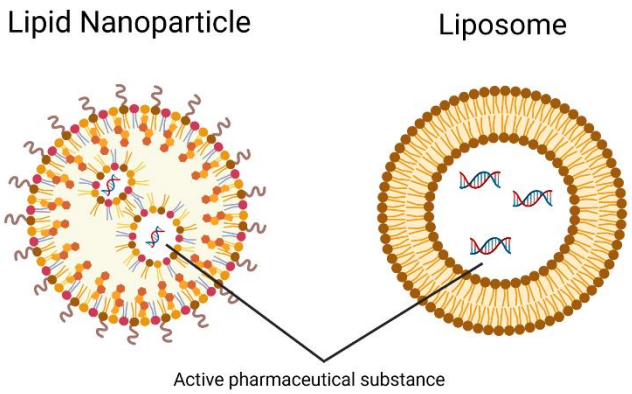


*Figure 5 - Lipid nano particle and liposome structure. Created with BioRender.com*

The intrinsic effect of the liposomes or LNPs on the immune system can have various effects. The stimulation or suppression of the immune response is determined by the size, charge, lipid composition and surface chemistry of the particle[62,63]. The particles are often first picked up by phagocytic cells, such as macrophages, of the immune system[21]. There are have been contradicting report on whether negative or positive charge is optimal for macrophage uptake[64–66]. Due to different compositions of lipid carriers used it is difficult to find the ideal charge optimal for the purpose of osteogenesis. If or how the intrinsic effect on the immune response influences bone formation is yet unknown. With the immense impact of the first immune response on bone formation, it is expected that intrinsic immune modulation of lipid carrier can be of influence the induction of bone formation.

## 2.3 Lipid based hydrogel coating

For a clinical application of lipid carrier systems, the immobilization or coating through a hydrogel offers various advantages. The mesh network acts as a temporal scaffold which indirectly immobilizes the lipid carrier[67]. The inherent properties of hydrogels allow for efficient encapsulation and liberation in a controlled manner. A local release of the active substance can be realized. Which is required in this research project, as the local inflammatory response is targeted for modulation. Another advantage is the high control over the release profile of the lipid carrier from the hydrogel, hydrogel degradation can be controlled by polymer concentration or degree of crosslinking[68]. Biopolymer based hydrogels offer certain advantages in biocompatibility and biodegradation. Gelatin methacrylate (GelMa) is a versatile hydrogel which can be used in various biomedical applications[69,70]. The natural components provide a cell friendly environment for proliferation and differentiation. Furthermore is GelMa ideal for incorporating a lipid carrier. Incorporation of the lipid carrier inside a GelMa hydrogel allows for easier storage. The hydrogel does not need to stay hydrated while being stored, it can be dried and rehydrated without losing it's stability or release the lipid carrier (Figure 6) [60].

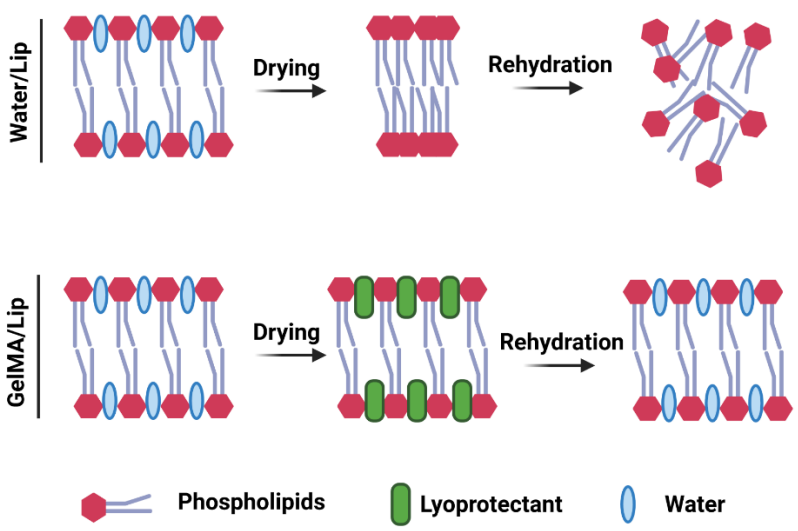


*Figure 6 - GelMa and other hydrogels in the process of drying. Many hydrogels need to be hydrated to remain stable, GelMa as hydrogel has a lyoprotective function. Created in biorender.com*

This hydrogel-lipid carrier mixture is not a coating attached to a surface. To achieve attachment to the surface of an implant various techniques can be used. The most promising method seems to be the spraying method[71]. A novel technique is the electro spray coating technique. This technique does not require rigorous processing conditions, has a high level of adhesion and allows a precise control over coating thickness[72]. The electro spraying method disperses small charged droplets unto the substrate through electrostatic forces. By applying a high voltage, a high electric field is created between the syringe and the collector (Figure 7)[60].The electric field exceeds the surface tension of large droplets, causing the droplets to break into nano sized droplets. The opposite charge at the collector causses the droplets to deposit on the surface of the substrate. A unified microgel layer is formed on the substrate due to evaporation and Coulombic fission of the droplets[73].

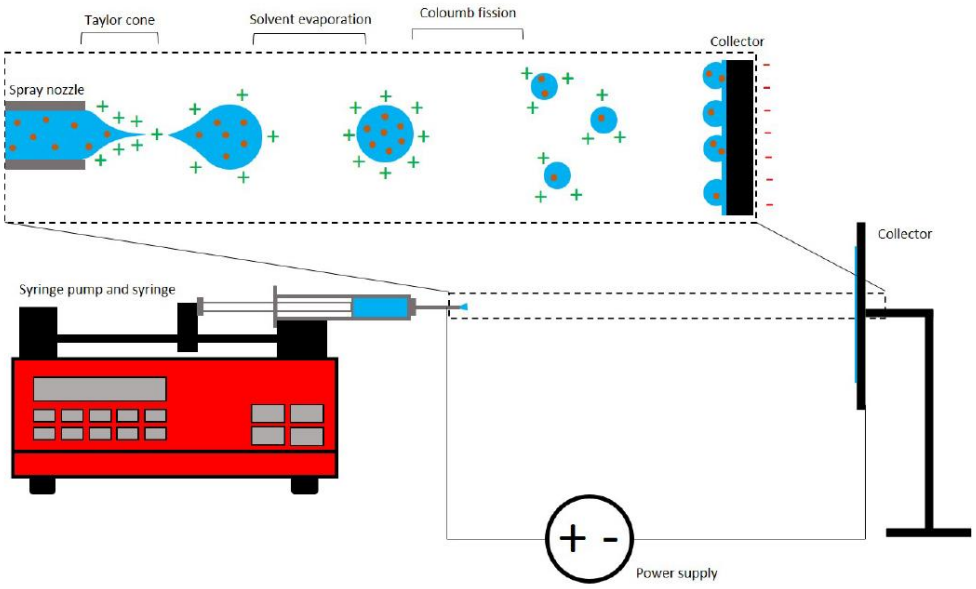


*Figure 7 – Electro spraying method. The lipid-GelMa mixture is driven through the nozzle of a syringe by a syringe pump. The generated high electrical field causes solvent evaporation and Coulomb fission in the droplets, this breaks the droplets into nano sized droplets. The nano droplets form a unified microgel layer at the collector due the to opposite charge of the collector. Created by biorender.com*

# 3. Material and method

## 3.1 Intrinsic osteo-immunomodulatory effect of liposomes

### 3.1.1 materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (**DPPC**), 1,2-dioleoyl-3-trimethylammonium-propane (**DOTAP**), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (**DPPG**), L-a-phosphatidylethanola- mine-N-(Lissamine Rhodamine B sulfonyl) (**Rho-PE**) were purchased form Avanti Polar Lipids (Alabaster, AL, USA), Fluorescein isothiocyanate-dextran (60/76kD) (**FITC**), Cholesterol (**CH**) was purchased from Sigma-Alrich (St. Louis, MO, USA). Gelatin type A from porcine skin (Sigma-Aldrich) was used as hydrogel. Solid Ti implants (diameter 8 mm; thickness 3 mm) were printed by selective laser melting.

### 3.1.2 Study design

This study focusses on the intrinsic osteo-immunomodulatory effects of cationic and anionic liposomes. Influencing the early inflammatory response in favour of bone formation is the focus in this research. The first part of the research consists of the synthesis of the liposomes. The anionic liposome had been formulated in previous research[60]. For the cationic liposome DOTAP was used to introduce a positive zeta-potential, as described in other research[74,75]. It was unknown which molar fraction was optimal for the cationic liposome, so different formulations were tried. Both liposomes were synthesised with the thin film method.

In favour of clinical translation, it is important that the liposomes are able to be applied as a stable coating on orthopedic implants. The feasibility of the liposome coating on titanium implants to influence osteo-immunomodulation is studied. The liposomes embedded in a GelMa hydrogel are electro sprayed on titanium implants as seen in Figure 8. Coated implants were incubated at 37 °C for 3 days in cell culture media, the media was collected every day and stored for further use.

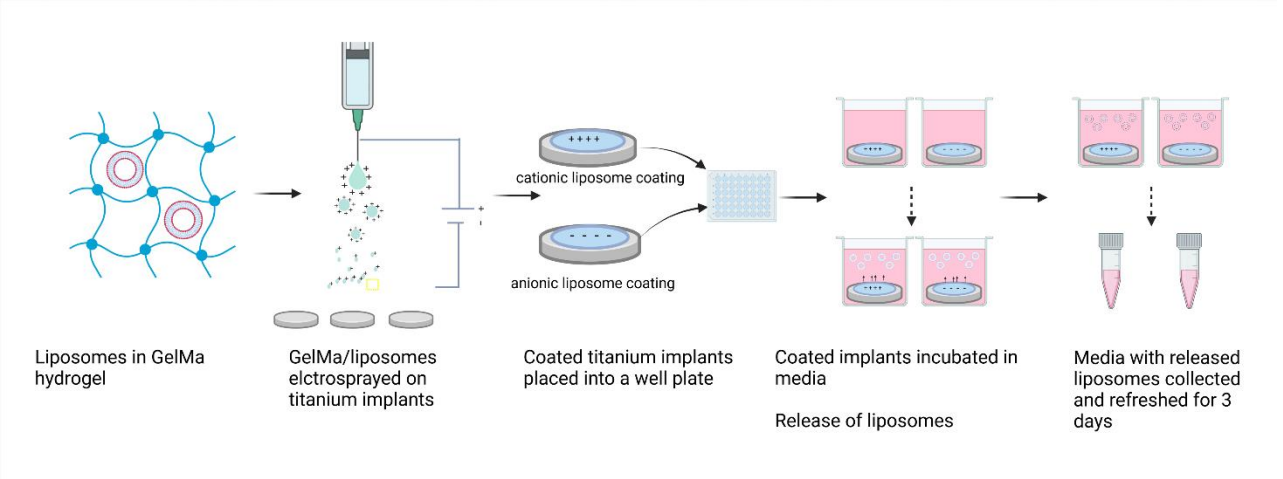


*Figure 8 – Cationic and anionic liposomes embedded in GelMa hydrogel coated on titanium implants. The coated implants are incubated in cell culture medium at 37 °C for 3 days. Each day the culture media is collected and new culture media is added to the well. The collected media of each day is stored and used in other experiments. Created by Biorender.com*

The different groups which were used can be seen in Table 1

| | | |
|---|---|---|
| **Anionic** | Liposome | 3 mM |
| | | 1.5 mM |
| | | 0.15 mM |
| | | 0.03 mM |
| | Released liposome media | Released day 1 |
| | | Released day 2 |
| | | Released day 3 |
| **Cationic** | Liposome | 3 mM |
| | | 1.5 mM |
| | | 0.15 mM |
| | | 0.03 mM |
| | Released liposome media | Released day 1 |
| | | Released day 2 |
| | | Released day 3 |

*Table 1 – Different groups used for the study of the intrinsic osteo-immunomodulatory effects of liposomes. Cationic and anionic liposomes were used in different concentrations. Liposomes released during the incubation of coated titanium implants were used. The release of the first, second and third day were selected for this study.*

The second part of the research focusses on the immunomodulatory effect of the liposomes. Additionally, the ability of the released liposomes from the coating is studied for their capacity to induce an immune response. The immunomodulatory effect will be analysed by the cytokine expression of murine and human macrophages, as well as Nuclear factor-κB (NF- κB) activation in murine cells. Macrophages were chosen as highlighted immune cell to mimic the early inflammation response. These immune cells are one of the first cells to be recruited toward the area of implantation or injury during the endogenous inflammation reaction[35]. The cytokine response of macrophages is pivotal in directing the further inflammation reaction. The choice to only include macrophages as immune cells was made in order to create a more controlled environment for analysis. The effects seen can be assigned solely to the functioning of macrophages. Human macrophages were handled as seen in Figure 9. Murine macrophages were handled in a similar manner, without the need to differentiate them for monocytes.

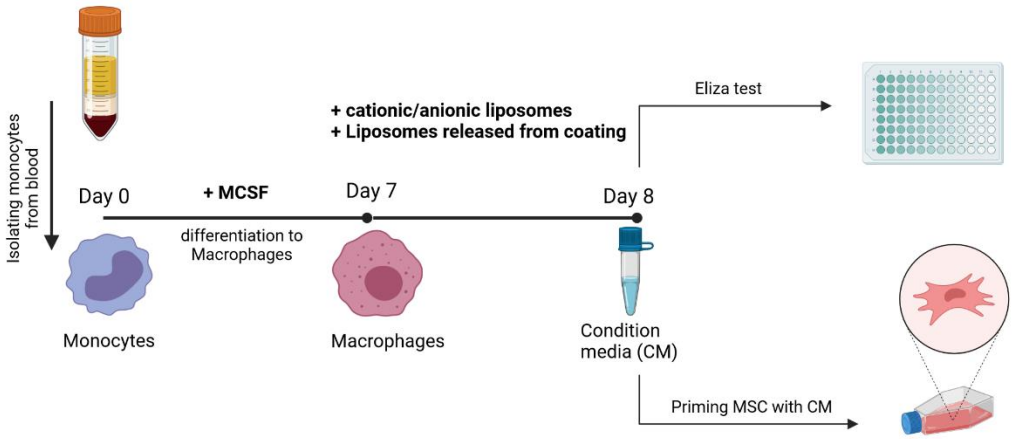


*Figure 9 –Macrophage experimental set-up. Human monocytes were isolated from the blood and were differentiated in macrophages by addition of M-CSF for 7 days. The macrophages were stimulated directly with the cationic/ anionic liposomes or through culture medium with cationic/anionic liposomes released from the coating for 24 hours. The media of the stimulated macrophages was collected for ELIZA test and for stimulation of human MSCs. Created by biorender.com*

The third part of the research focusses on the capacity for osteogenic induction. The initial inflammation phase during bone regeneration is highly influential for the eventual outcome of bone formation[15]. The cross-talk between human MSCs and macrophage is an important factor. The manner of macrophage activation influences the osteogenic capacities of MSCs[76]. This initial phase is mimicked in the current set-up, the capacity of liposomes to influence this phase in favour of osteogenesis is analysed (Figure 10). A similar set-up has been used in previous research[49]. Human MSCs, the progenitor cells of bone forming osteoblasts, are crucial targets for bone regeneration. The ALP activity can serve as an early marker for osteogenic potential of human MSCs[54]. In the indirect set-up, the effect of liposomes through macrophage stimulation on ALP is analysed. The macrophage media, after 24 hours of stimulation by liposomes, will be added to the human MSCs. In the direct set-up, the direct influence of the liposomes on human MSC ALP activity is analysed. The liposomes are added directly to the human MSCs.

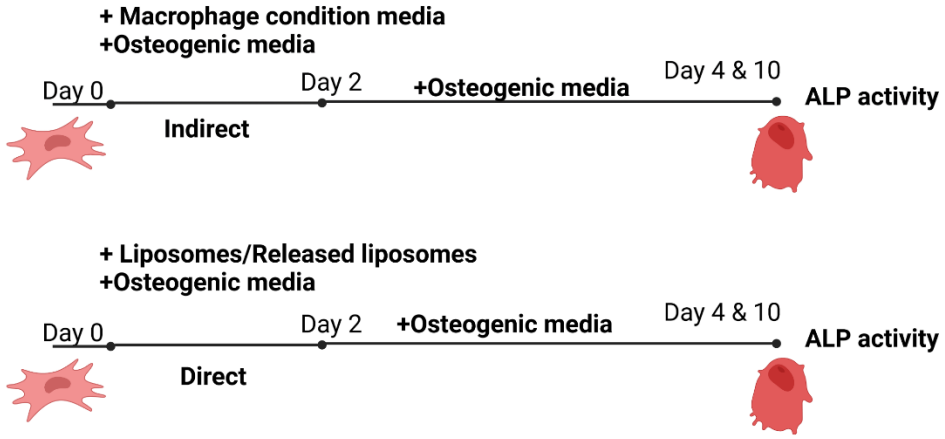


*Figure 10 – Experimental set-up for early osteogenic marker (ALP activity). Indirect: Human MSCs are stimulated with signals released from macrophages (which have been stimulated for 24 hours with liposomes/released liposomes) together with osteogenic media for 2 days. Then the media is refreshed with osteogenic media without stimulation. The ALP activity is then determined for 2 different time points(4 days and 10 days). Direct: Human MSCs are stimulated with liposomes and released liposomes together with osteogenic media for 2 days. Then the media is refreshed with osteogenic media without stimulation. The ALP activity is then determined for 2 different time points(4 days and 10 days). Created by biorender.com*

### 3.1.3 Liposome preparation

The anionic liposome was composted of DPPC/CH/DPPG in molar ratio 63/30/7, the cationic liposome was composted of DPPC/CH/DOTAP in molar ratio 61/31/8. The thin film preparation method as reported before was used to create the liposomes[77]. The preparation is the same as described in previous research[60]. A round bottom flask was used to make a lipid film by dissolving the lipids in chloroform and methanol mixture (2:1) and evaporating the organic solvents using a rotary evaporator at 60 °C and reduced pressure. The formed dry film was flushed with nitrogen gas to remove all traces of organic solvent for 10 minutes. Rhodamine-PE containing liposomes consisted of 0.1 mole% of Rho-PE with respect to total lipid content. The lipid film was hydrated in HEPES buffer saline (HBS) pH 7.4 at 50 °C with a volume that resulted in a theoretical lipid concentration of 30 mM liposome. FITC-Dextran-60/76k containing liposomes contained 10 mg/mL of FITC-Dextran-70k in the hydration buffer. After hydration, the liposome suspension was extruded 10 times through a extruder using an Avanti Mini-extruder through 2x400 nm polycarbonate filters at room temperature. Next, three rounds ultracentrifugation (Type 70.1 Ti rotor) were performed for 50 minutes at 55.000 RPM and 4 °C as a purification step. Finally, after the final ultracentrifugation run, the supernatant was removed and replaced by a volume of HBS that resulted in a theoretical lipid concentration of 30 mM liposomes.

### 3.1.4 Liposome characterization

The hydrodynamic radius and polydispersity index (PDI) were measured by dynamic light scattering (DLS), using Nano-S (Malvern Instruments, Malvern, UK) under an angle of 173. The zeta-potential of the particles were measured using a Doppler electrophoresis a Zetasizer Nano-Z (Malvern Instruments, Malvern, UK). DLS and zeta-potential measurements were measured with 2.5% (v/v) final liposome dispersed in 10 mM HEPES buffer pH 7.4. For verification of intactness of the Liposomes after electro spraying, the colocalization of the fluorescence signals produced by Lips encapsulating hydrophilic cargo (dextran-FITC) in the aqueous core and a lipophilic dye (Rhodamine-PE, Rh) in the shell was studied.

### 3.1.5 Electro spray coating

GelMa hydrogels were synthesized by adding 0.6 g of methacrylic anhydride per gram of gelatin (10% w/v in PBS). The GelMa solution was dialyzed against deionized water to remove unreacted methacrylic anhydride. For electro spraying, 6% GelMa solution was prepared with the addition of 0.5% irgacure (w/v) as a photo initiator. The custom-built electrospray setup consisted of a high voltage power supply (Heinzinger LNC 30000) and a syringe pump, allowing control over voltage (16 kV) and flow rate (0.5 ml/h). Electro sprayed droplets were deposited onto titanium implants at a distance of 10 cm from the needle tip. The samples were UV-irradiated for 6 minutes for photo-crosslinking. 50 (v/v)% of liposome/GelMA (15mM Lips/60 mg/ml GelMA) were electro sprayed on implant.

### 3.1.6 Immune stimulation macrophages

Cytokine production by human and murine macrophages was measured to quantify the immunomodulatory properties of liposomes. Human Monocytes were isolated from peripheral blood (n=2, Mini Donor Dienst, UMC Utrecht). CD14+ monocytes were purified by MACS® technology (Miltenyi). Cells were seeded in RPMI medium containing 1% FBS and 1% PenStrep at a density of 150,000 cells/well in 96-well plates, and differentiated into macrophages for 7 days in the presence of 25 ng/ml M-CSF (Peprotech). RAW 264.7 murine macrophages were seeded at a density of 50,000 cells per well in DMEM medium containing 10% FBS and 1% Penstrep.

Macrophages were stimulated for 24 h with different liposome suspensions and released media from coated implants, using lipopolysaccharide (LPS, from Escherichia coli O111:B4, Sigma) at 100 ng/ml as a positive control. After 24 h the supernatant was collected and stored in -80 $^{o}$C. The cytokine concentrations in the supernatant were determined by ELISA (DuoSet®, RD Systems).

The NF-κB activation of RAW-Blue™ cells was analysed. The RAW-Blue™ cells stably express a ALP gene inducible by NF-κB transcription factors. The RAW-Blue™ cells were seeded at a density of 100,00 cells per well in a 96-well plate in expansion media (DMEM containing 10% FBs and 1% PenStrep). The RAW-Blue™ cells were stimulated with the different groups for 24 hours. RAW-Blue™ cells without stimulation served as a negative control, while RAW-Blue™ cells with 100ng/ml LPS served as a positive control. 20 ul supernatant was collected from each well. ALP was measured in the supernatant by the conversion of the p-nitrophenyl phosphate liquid substrate system (pH = 9.6) (SigmaFast p-nitrophenyl phosphate tablets, Sigma-Aldrich). The absorbance was measured at 405 nm and corrected at 655 nm (Bio-Rad, Hercules, CA, USA).

### 3.1.7 Osteogenic assay human MSCs

To test the effect of liposomes on early osteogenic differentiation, human MSCs were seeded at a density of 5,000 cell pr well in a 96-well plate. Cells were cultured in osteogenic differentiation medium (expansion medium supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone), in absence or presence of liposomes or conditioned media form macrophages

(Ratio osteogenic media/conditioned macrophage media – 8/1). As a negative control group human MSCs were also cultured in expansion media. For the direct assay LPS was added as a positive control at as concentration of 100ng/ml for the first 2 days. The medium was refreshed twice a week, after 4 and 10 days the cells were collected for ALP activity.

For intracellular ALP activity quantification, cells were lysed in 0.2% (v/v) Triton X-100/PBS for 30 min. ALP activity was measured by the conversion of the p-nitrophenyl phosphate liquid substrate system (pH = 9.6) (SigmaFast p-nitrophenyl phosphate tablets, Sigma-Aldrich). The absorbance was measured at 405 nm and corrected at 655 nm (Bio-Rad, Hercules, CA, USA). The cell lysate was also used to determine the DNA content with the Quant-It PicoGreen kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The ALP/DNA was normalized for the control not receiving any liposomes.

## 3.2 Osteo-immunomodulatory effect of PAMPs encapsulated in LNPs

### 3.2.1 materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (**DPPC**), 1,2-dioleoyl-3-dimethylammoniumpropane (**DODAP**), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (**DPPG**), L-a-phosphatidylethanola- mine-N-(Lissamine Rhodamine B sulfonyl) (**Rho-PE**) were purchased form Avanti Polar Lipids (Alabaster, AL, USA), Cholesterol (**CH**) was purchased from Sigma-Alrich (St. Louis, MO, USA). High molecular weight polyinosinic-polycytidylic acid (**Poly(I:C)**) and CpG C oligonucleotide (**CpG C ODN**) were purchased from Invivogen (San Diego, CA, USA). Methyl methacrylate monomer (**MMA**) purchased from Merck. Nonylphenyl-polyethyleneglycol acetate (**Plastoid N**) and Benzoyl Peroxide were purchased from Sigma. **BCP** implants were MagnetOs: 65–75% Tri-Calcium Phosphate and 25–35% Hydroxyapatite (Kuros Bioscience) with a diameter of 9mm.

### 3.2.2 Study design

This study focusses on the osteo-immunomodulatory effects of CpG C and Poly(I:C) encapsulated in LNPs. Influencing the early inflammatory response in favour of bone formation is the focus in this research. The encapsulation of the PAMPs in LNPs is though to improve the transfection of the PAMPs into the cells, it will be assessed if there is an increased osteogenic effect due to the encapsulation. The formulation of LNPs and their encapsulation of CpG C and Poly(I:C) has been described in previous research[60].

The different groups can be seen in Table 2.

| | | |
|---|---|---|
| **Empty - LNP** | 10 ug/ml* | *The volume of empty-LNP used for each concentration was the same as the volume used for LNPs encapsulating PAMPs for each concentration. |
| | 1 ug/ml* | |
| | 0.1 ug/ml* | |
| **CpG C encapsulated in LNP** | 10 ug/ml** | **The concentration refers to the concentration of CpG C which is encapsulated. |
| | 1 ug/ml** | |
| | 0.1 ug/ml** | |
| **Poly (I:C) encapsulated in LNP** | 10 ug/ml*** | ***The concentration refers to the concentration of Poly(I:C) which is encapsulated. |
| | 1 ug/ml*** | |
| | 0.1 ug/ml*** | |
| **Free form CpG C** | 10 ug/ml | |
| | 1 ug/ml | |
| | 0.1 ug/ml | |
| **Free form Poly (I:C)** | 10 ug/ml | |
| | 1 ug/ml | |
| | 0.1 ug/ml | |

*Table 2 - Different groups used for the study of osteo-immunomodulatory effects of LNPs encapsulating PAMPs.*

The first part of the *in vitro* research will be the assessment of the immunomodulatory effect of the PAMPs. The experimental set-up is similar as described in section 3.1.2. The cytokine expression of human macrophages will be analysed (Figure 11). Furthermore, will the direct and indirect effect of LNPs on human MSC ALP activity be assessed.

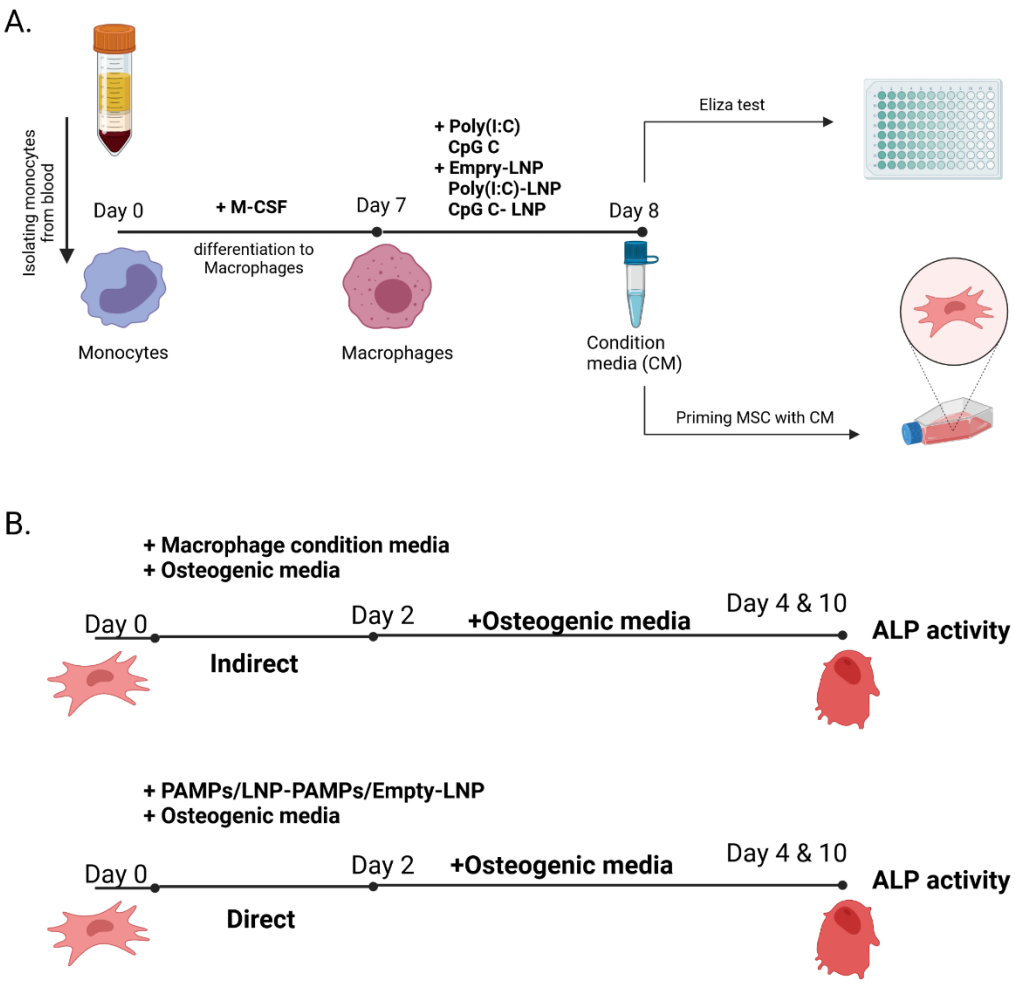


*Figure 11 – Experimental set-up for in vitro assessment of osteo-immunomodulatory effect of LNPs encapsulating PAMPs. A: Human monocytes were isolated from the blood and were differentiated into macrophages by addition of M-CSF for 7 days. The macrophages were stimulated directly with Empty-LNP, CpG C-LNP, Poly(I:C)-LNP, free form CpG C and free form Poly(I:C) for 24 hours. The media of the stimulated macrophages was collected for ELIZA test and for stimulation of human MSCs. B: Experimental set-up for early osteogenic marker (ALP activity). Indirect: Human MSCs are stimulated with signals released from macrophages (which have been stimulated for 24 hours with Empty-LNP, CpG C-LNP, Poly(I:C)-LNP, free form CpG C and free form Poly(I:C)) together with osteogenic media for 2 days. Then the media is refreshed with osteogenic media without stimulation. The ALP activity is then determined for 2 different time points(4 days and 10 days). Direct: Human MSCs are stimulated with Empty-LNP, CpG C-LNP, Poly(I:C)-LNP, free form CpG C and free form Poly(I:C) together with osteogenic media for 2 days. Then the media is refreshed with osteogenic media without stimulation. The ALP activity is then determined for 2 different time points(4 days and 10 days). Created by biorender.com*

The *in vivo* part of this research consist of a rabbit study. The *in vivo* effect of all the groups is assessed in a intramuscular rabbit model. BCP implants were prepared as can be seen in Table 3. The LNPs were embedded inside a GelMa hydrogel for increased stability during storage and controlled release in the rabbit.

| Group name | Components | | | Number of samples |
|---|---|---|---|---|
| **PBS** | BMP-2 | PBS | | 5 |
| **GelMa** | BMP-2 | GelMa | | 21 |
| **CpG C** | BMP-2 | GelMa | CpG C | 8 |
| **Poly (I:C)** | BMP-2 | GelMa | Poly (I:C) | 6 |
| **LNP** | BMP-2 | GelMa | Empty-LNP | 7 |
| **LNP/CpG C** | BMP-2 | GelMa | CpG C-LNP | 7 |
| **LNP/Poly(I:C)** | BMP-2 | GelMa | Poly (I:C)-LNP | 8 |

*Table 3 – Groups used in the in vivo rabbit study.*

23 rabbits were used in the study, where each rabbit had 3 constructs intramuscularly implanted (Figure 12). Every rabbit had one GelMa implant as control, the other groups were divided randomly (Appendix B).

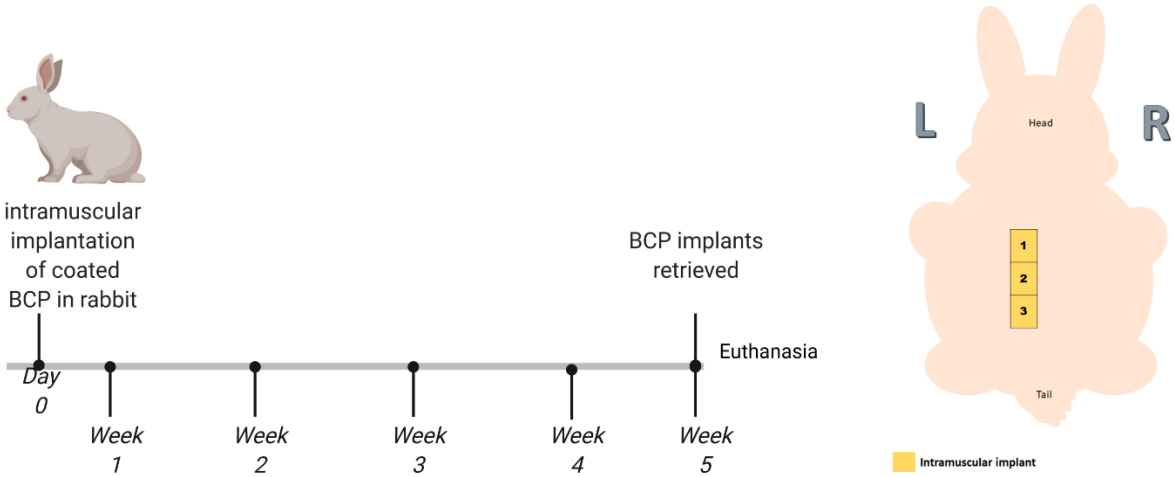


*Figure 12 – Timeline for the in vivo rabbit study and implantation site for intramuscular implants. Created by biorender.com*

The implants were retrieved after 5 weeks from the rabbit (Figure 12). The retrieved constructs were sectioned, stained and bone formation was quantified (Figure 13). The bone area % is defined as the percentage of bone in the available pore space.

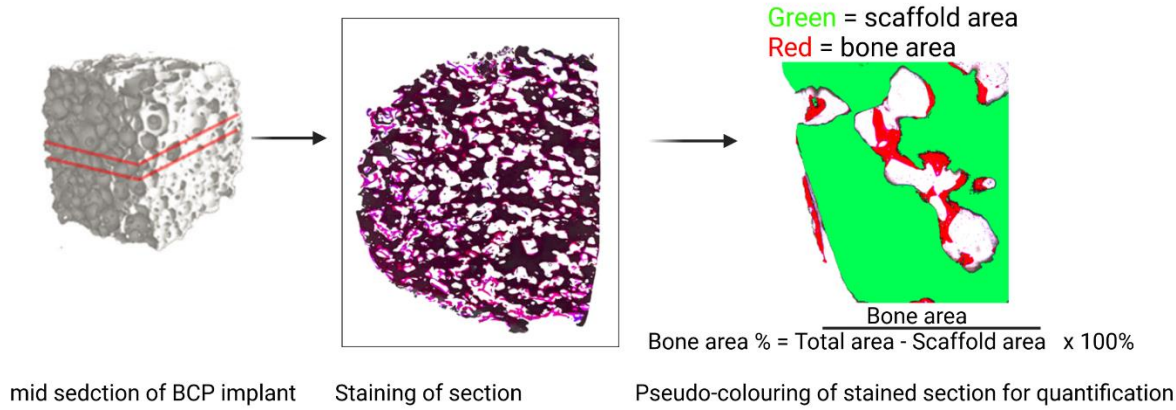


*Figure 13 –Analysis of Retrieved scaffold procedures. Embedded retrieved scaffolds are sectioned and stained. The staining is pseudo-coloured for quantification of bone are %. Image adapted from "Interleukin 17 enhances bone morphogenetic protein-2-induced ectopic bone formation" Croes, M., Kruyt, M.C., Groen, W.M.* et al. Sci Rep ***8,*** *7269 (2018). doi-org.proxy.library.uu.nl/10.1038/s41598-018-25564-9* [78]

### 3.2.3 Lipid nano particle preparation

All LNPs were composed of DPPC/DODAP/CH/DPPG in molar ratio 38/25/30/7. LNPs were create by the ethanol dilution methos as described in previous work[79]. First, nucleic acid solution was prepared by combining nucleic acid stock solution with 67 mM citrate buffer (pH 4.0) and distilled deionized water. The final condition was 40 mM citrate buffer and the nucleic acid concentration of 200ug/ml. Lipid stock solutions were in 90% ethanol with 20 mM total lipids. The lipid/ethanol solution was heated under hot water and mixed firmly before addition. The lipid/ethanol solution was added dropwise to the nucleic acid solution using a 1 mL syringe and a 0.45x22 mm needle, while being constantly stirred. Equal volume of both lipid/ethanol and nucleic acid solution were used. Upon initial particle formation, the mixture was further diluted with the same volume of 300 mM NaCl and 20 mM citrate buffer pH 4.0 by the same previous dropwise addition. The diluted vesicles were incubated at room temperature for one hour before purification. The purification of lipid nanoparticles was performed by dialysis against 20 mM citrate buffer pH 4.0 containing 150 mM NaCl for 2 hours to remove the ethanol in a 10K Dialysis Cassette (MWCO Slide-A-Lyzer G2, Life Technologies) at 4 °C. The dialysis solution was refreshed each hour. The mixture was further dialyzed overnight against HBS (pH7.4, 4 °C) to neutralize the DODAP. All dialysis buffers were 1000 times more than the volume of the sample. Next, three rounds ultracentrifugation (Type 70.1 Ti rotor) were performed for 50 minutes at 55.000 RPM and 4 °C to was away unencapsulated nucleic acids. Finally, after the final ultracentrifugation run, the supernatant was removed and replaced by a volume of HBS that resulted in a theoretical lipid concentration of 30 mM lipids.

### 3.2.4 LNP characterization

The hydrodynamic radius and polydispersity index (PDI) were measured by dynamic light scattering (DLS), using Nano-S (Malvern Instruments, Malvern, UK) under an angle of 173. The zeta-potential of the particles were measured using a Doppler electrophoresis a Zetasizer Nano-Z (Malvern Instruments, Malvern, UK). DLS and zeta-potential measurements were measured with 2.5% (v/v) final LNPs dispersed in 10 mM HEPES buffer pH 7.4.

### 3.2.5 Immune stimulation macrophages

Cytokine production by human macrophages was measured to quantify the immune-modulatory properties of LNPs. Monocytes were isolated from peripheral blood (n=2, Mini Donor Dienst, UMC Utrecht). CD14+ monocytes were purified by MACS® technology (Miltenyi). Cells were seeded in RPMI medium containing 1% FBS and 1% PenStrep at a density of 150,000 cells/well in 96-well plates, and differentiated into macrophages for 7 days in the presence of 25 ng/ml M-CSF (Peprotech). Macrophages were stimulated for 24 h with different LNP suspensions, using lipopolysaccharide (LPS, from Escherichia coli O111:B4, Sigma) at 100 ng/ml as a positive control. After 24 h the supernatant was collected and stored in -80 °C. The cytokine concentrations in the supernatant was determined by ELISA (DuoSet®, R&D Systems).

### 3.2.6 Osteogenic assay human MSCs

Human MSCs were seeded at a density of 5,000 cells/well in a 96-well plate. Cells were cultured in osteogenic differentiation medium (expansion medium supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone), in absence or presence of liposomes or conditioned media form macrophages. As a negative control group human MSCs were also cultured in expansion media. For the direct assay LPS was added as a positive control at as concentration of 100ng/ml for the first 2 days. The medium was refreshed twice a week, after 4 and 10 days the cells were collected for ALP activity.

### 3.2.6 *In Vivo* study

The BCP implants were coated according to the concentrations seen in Table 4, components were dissolved in HBS. Firstly 50 ul of BMP-2 (100ug/ml) was added directly by pipette to the BCP implant. Secondly 150 ul of the rest mixture was added directly by pipette to the BCP implant, after which the implants were crosslinked by UV exposure (Bluepoint) for 6 minutes. The implants were prepared in the same week as the implantation. The implants were inserted intramuscular in rabbits and removed after 5 weeks for analysis of bone formation.

| Conditions of BCP implants | |
|---|---|
| *Drug concentration (ug/ml)* | 1 |
| *GelMa concentration (wt%)* | 6 |
| *Irigacure concentration (wt%)* | 0.5 |
| *BMP-2 concentration (ug/ml)* | 25 |
| *Total volume (ul)* | 200 |

*Table 4 – BCP implant conditions.*

When the implanted constructs were retrieved from the rabbits, they were firstly fixed in 10% formalin on a shaker. Then the constructs were dehydrated through in a ethanol series. The constructs were dehydrated two times in 70%, two times in 96% and two times in 100% EtOH for respectfully 2 days, 2 days, and 3 days. Subsequently, the constructs were embedded in MMA in three steps. Firstly for 5 days in MMA-1 solution (MMA monomer without additives). Secondly for 5 days in MMA-2 solution (MMA with benzoyl peroxide). Lastly for 5 days in MMA-3 solution (MMA with benzoyl peroxide and Plastoid N) . After embedding, the constructs were cut in approximately 40 µm-thick sections with a sawing microtome (Leica, Nussloch, Germany) and were stained with basic fuchsin and methylene blue. Images of the sections were made using the microscope(Leica Thunder imaging system)Two mid- sections from each implant were pseudo-coloured using Adobe Photoshop (Adobe Systems, San Jose, USA) for quantification of bone growth. The percentage of bone in the available pore space (bone area %) was quantified. The mean value of the two sections was used as a result per construct.

# 4. Results and discussion

## 4.1 intrinsic osteo-immunomodulatory effect of liposomes

### 4.1.1 Liposome characterization

In order to analyse the intrinsic osteo-immunomodulatory effect of liposomes, two opposite charged liposomes were created. The anionic liposome had been formulated in previous research[60]. Different molar fractions of DOTAP were tried in order to create the cationic liposome used in further experiments (Appendix A). The final formulation used was DPPC/CH/DOTAP in molar ratio 61/31/8. This formulation gave the desired positive zeta-potential at relatively low lipid costs. The characteristics of the cationic and anionic liposomes can be found in Table 5. DOTAP was responsible for the positive zeta potential found in the cationic liposomes. Furthermore was the size and zeta-potential of the cationic liposome dependant on the molar fraction of DOTAP (Appendix A). Corresponding with previous research, an increasing molar ratio of DOTAP decreased mean particle size and increased zeta-potential[74]. The Anionic liposomes showed a negative zeta potential due to DPPG incorporation. The Poly dispersity index (PDI) showed that the liposomes had a high monodisperse size distribution. PDI values lower than 0.3 are considered to be highly monodispersed[80]. The closer the value comes to 1, the higher the polydispersity of the mean particle size.

| | *Cationic liposome* | *Anionic liposome* |
|---|---|---|
| Mean particle size (nm) | 175 ± 7 | 249 ± 39 |
| PDI | 0.15 ± 0.02 | 0.23 ± 0.02 |
| Zeta-potential (mV) | 11 ± 1 | -35 ± 16 |

*Table 5 – Characteristics of cationic and anionic liposomes created by thin film method. The standard deviation was measured by the average of the samples.*

### 4.1.2 Osteo-immunomodulatory effects of liposomes *in vitro*

The different lipid compositions and charge of liposomes can have a variety of effects on specific cells, inducing various immune responses. Certain formulations of liposomes containing DOTAP have been reported to exhibited cytotoxic effects[81]. The effect of the liposomes used in this study on cell activity or DNA content of murine Raw cells and human monocytes can be seen in Figure 14. No significant effect on activity or DNA content of cells was seen. The cells stimulated by the charged liposomes had similar results as the unstimulated control group, indicating that for this research no influential cytotoxicity could be seen.

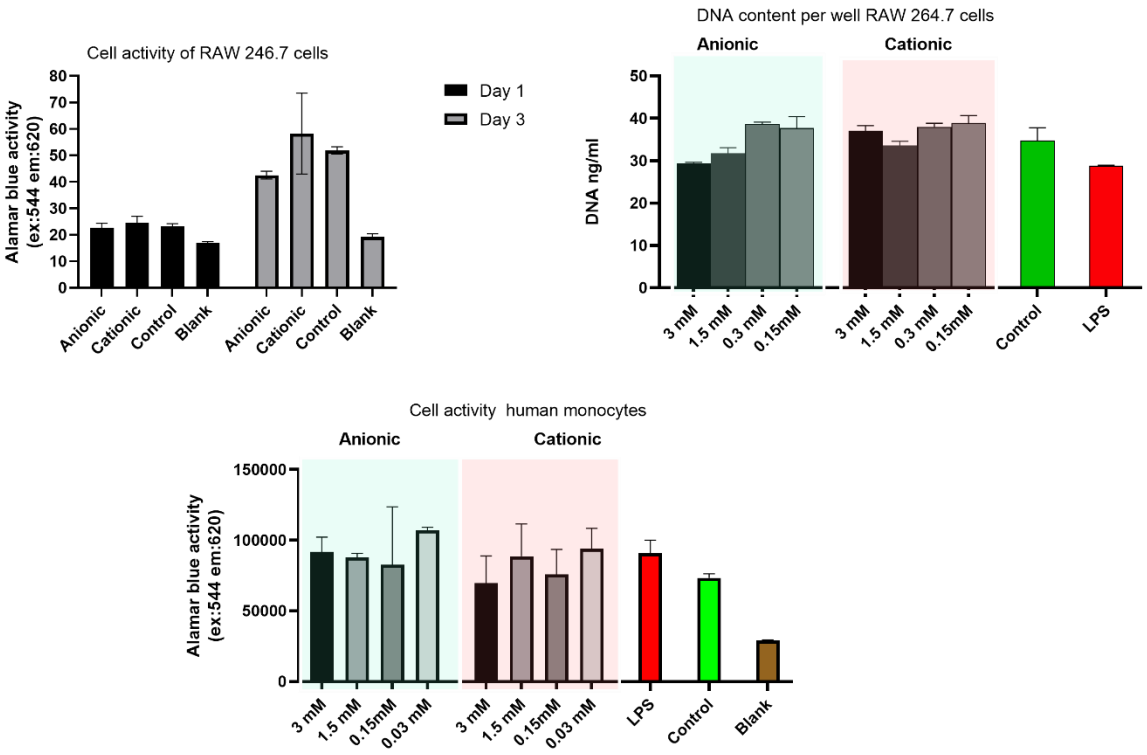


*Figure 14 – Cell activity of RAW 246.7 cells after one and three days of stimulation with cationic or anionic liposomes, the DNA content of RAW 264.7 cells after 24 hours of stimulation by different concentrations of cationic or anionic liposomes and cell activity of human monocytes after 24 hours of stimulation with cationic or anionic liposomes in different concentrations. The control group are unstimulated cells cultured in expansion media. The blank group is a negative control, it represent the signal without cells present. The LPS group is cells stimulated with LPS, mimicking an inflammatory response.*

The cytokine production of murine Raw cells was measured to asses inflammatory effects of the charged liposomes (Figure 15). The RAW 264.7 cells were exposed to decreasing concentrations of anionic and cationic liposomes, as well as cationic and anionic liposomes released from the coated titanium implants at day 1, 2 and 3, for 24 hours. The control group consists of RAW cells without stimulation and the LPS group serves as a positive control. LPS is a known activator the immune system[82,83]. Liposomes released from the coated titanium implant did not show a clear trend in inducing cytokine production. It is expected that most liposomes will be released in the first day and lower concentrations in the days thereafter. Thus a decreasing cytokine production would be expected ranging from day 1 to day 3 for liposomes released from the coating. However this could not be seen in the results. The low concentration of liposomes released or degraded products from the coating could explain the absence of a dose dependent trend in production of cytokines. The liposomes released from the coating did induce a inflammatory cytokine profile higher than the control group. A similar level of cytokine production was seen between anionic or cationic liposomes for direct addition and released from the coating. Directly added liposomes showed a dose dependant influence for the release of TNF-a, IL-6 and IL-10. For IL-8, only the highest concentration of 3 mM showed a slight in crease in cytokine production compared to the unstimulated control. Both cationic and anionic liposomes added directly to the cells showed to induce a dose dependent inflammatory response. TNF-a, IL-6 and IL-8 are pro-inflammatory cytokines secreted in early inflammation[84]. The release of these pro-inflammatory cytokines is important for the maintenance of inflammation and cell recruitment to the injury side. IL-10 production was higher in both high concentration cationic and anionic liposomes directly added compared to the LPS group. IL-10 has a anti-inflammatory function and is important in immunoregulation, balancing the immune reaction

for tissue regenartion[40]. Both charged liposomes seem to induce an similar inflammatory reaction in a dose dependant manner when added directly RAW 264.7 cells.

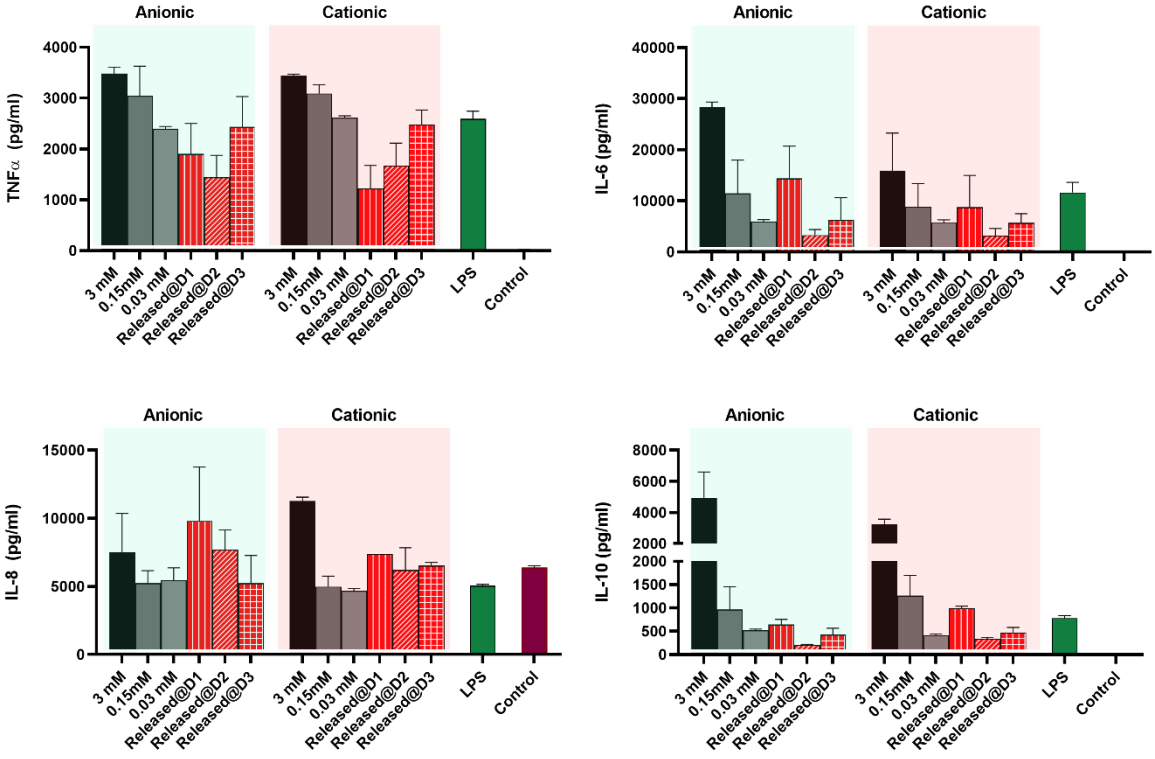


*Figure 15 – Cytokine production (TNF-a, IL-6, IL-8 and IL-10) of RAW 246.7 cells after 24 hours of stimulation with different concentrations of anionic and cationic liposomes, as well as cationic and anionic liposomes released from the coated titanium implants at day 1, 2 and 3. The control group represents the cytokine production without stimulation. The LPS group is the cytokine production after stimulation with LPS.*

The activation of the immune response by cationic and anionic liposomes seems to be mediated through NF-κB activation (Figure 16). Direct addition of cationic and anionic liposomes to RAW-blue™ cells lead to NF-κB activation in a dose dependant manner. 3 mM and 1.5 mM of cationic and anionic liposome show a similar activation of NF-κB as the LPS group. This indicates that the immune response initiated by the liposomes is mediated through -κB activation.

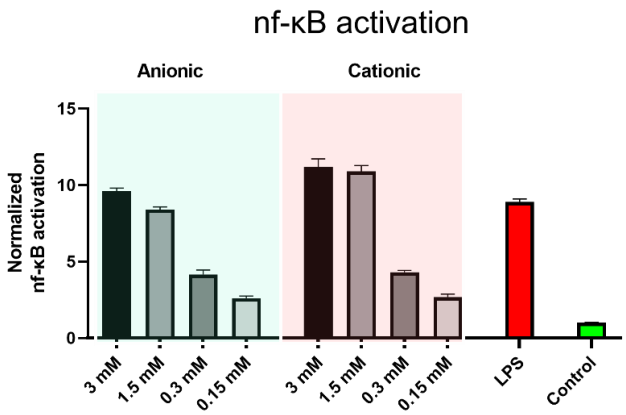


*Figure 16 – NF- κB activation in RAW-blue™ cells after 24 hours of stimulation with different concentrations of anionic and cationic liposomes. The control group represents cells without stimulation. The LPS group is stimulated with LPS, mimicking an inflammatory response.*

Cytokine production of human macrophages was measured after stimulation with a decreasing concentrations of anionic and cationic liposomes, as well as cationic and anionic liposomes released from the coated titanium implants at day 1, 2 and 3(Figure 17).

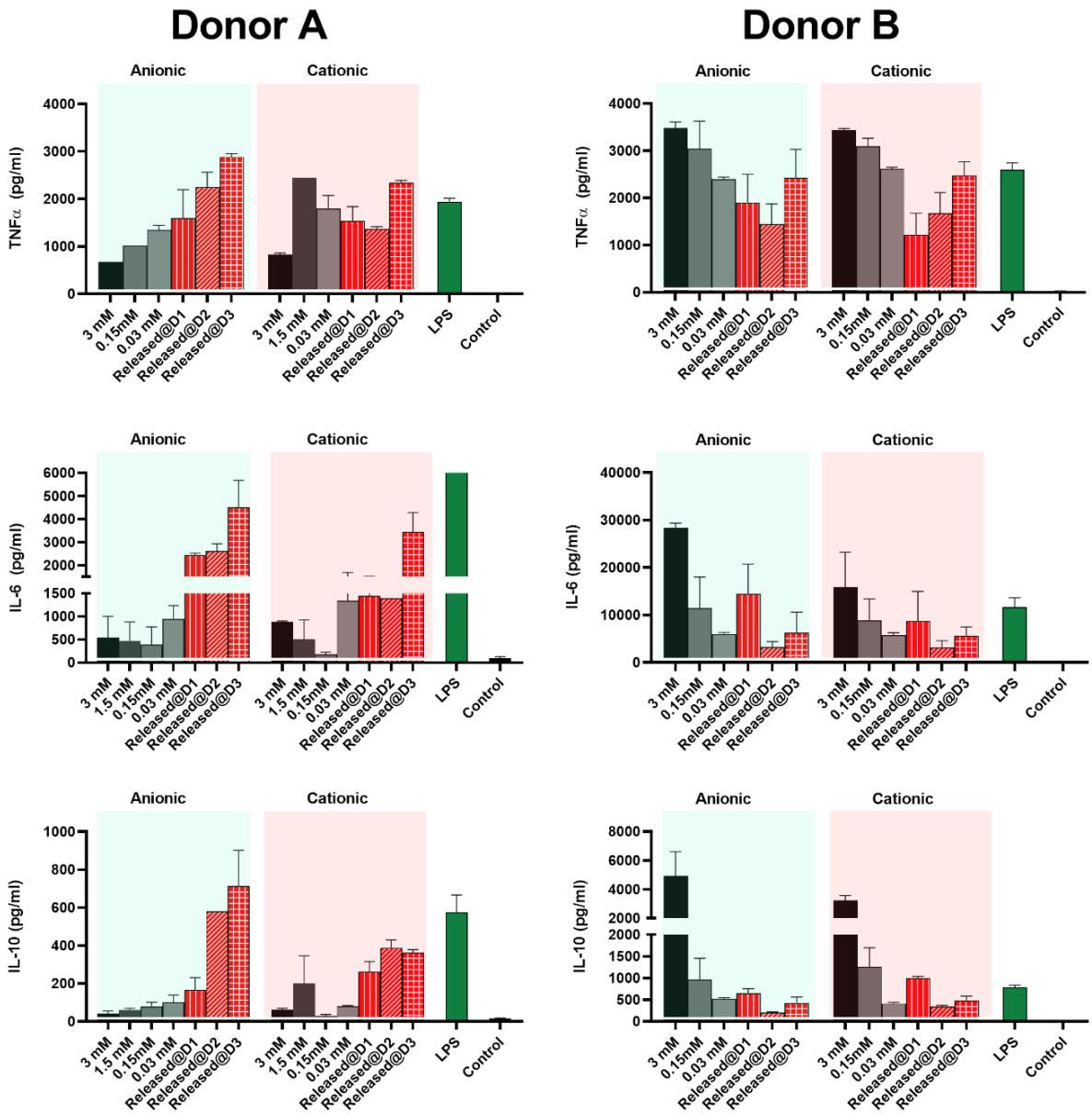


*Figure 17 - Cytokine production (TNF-a, IL-6 and IL-10) of human macrophages (2 donors) after 24 hours of stimulation with different concentrations of anionic and cationic liposomes, as well as cationic and anionic liposomes released from the coated titanium implants at day 1, 2 and 3. The control group represents the cytokine production without stimulation. The LPS group represents the cytokine production after stimulation with LPS.*

A high donor variation could be seen in the cytokine production. Furthermore was there no difference between the cationic or anionic liposomes. Only donor B showed a dose dependant cytokine production upon direct stimulation with the charged liposomes. Both donors did show a increased TNF-a production compared to the control group, indicating an inflammatory response. There was no clear dose dependant trend in the liposomes released from the coating group, which could again due to low concentration of liposomes released or degraded products from the coating having a effect. The cytokine production from the released group showed a higher or similar cytokine response as the directly added group, which is unexpected as lower concentrations of

liposome are expected to by present. The high cytokine production of the released liposomes could be due to the degraded GelMa hydrogel present in the media from the incubated coating. The GelMa used, has been shown to increase the production of TNF-a in human MSCs[85]. The endogenous endotoxins present in the GelMa could induce a immune reaction in the macrophages. The effect of the GelMa hydrogel on the cytokine production of macrophages should be tested to give a clear view of their influence. As of now it is unclear if the released liposomes cause the cytokine production or if the GelMa could induce the cytokine production.

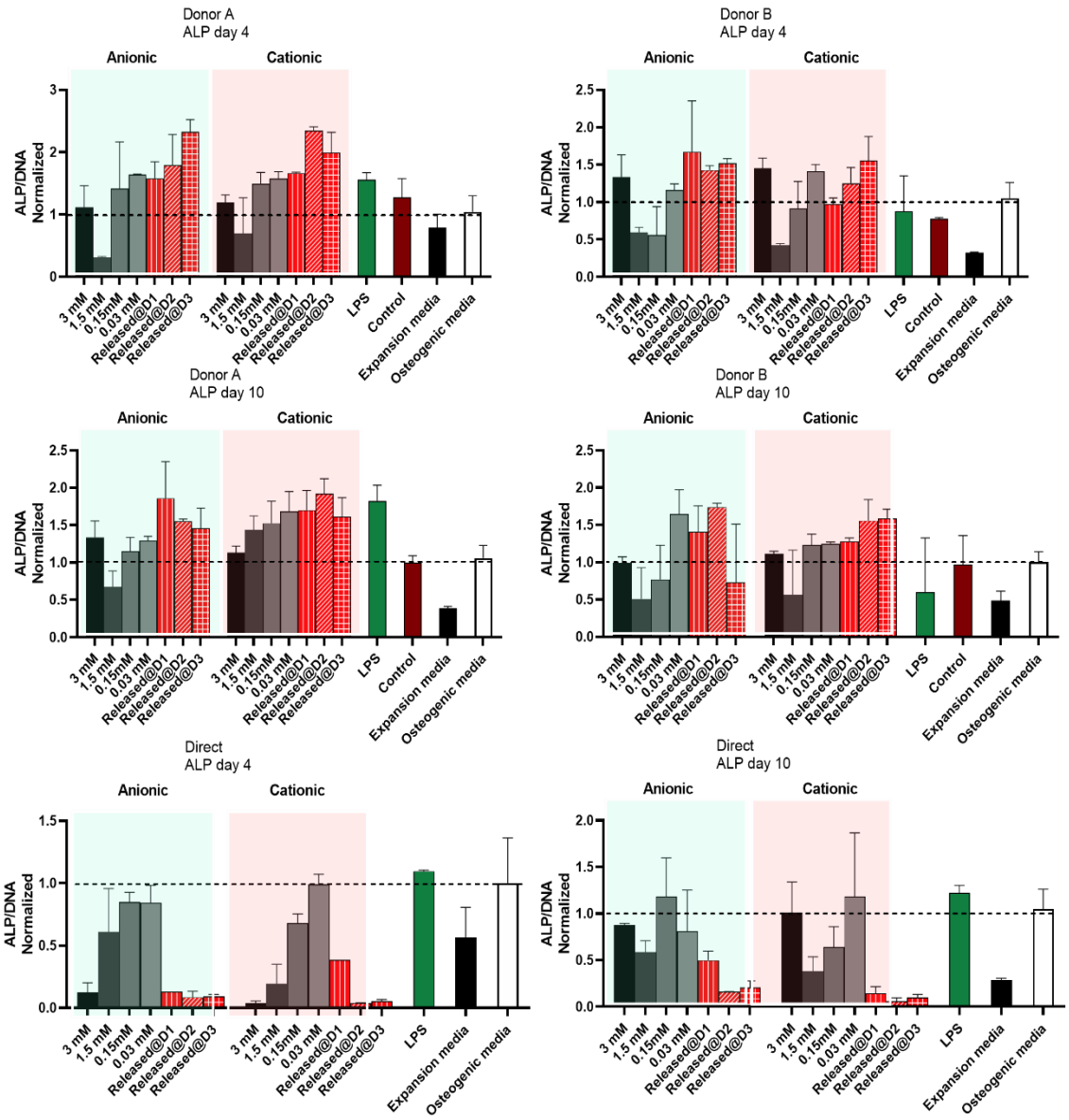


*Figure 18 - ALP activity of human MSCs at time points day 4 and day 10 after indirect (Macrophage conditioned media from Donor A and B) and direct stimulation. Indirect: LPS group represents human MSCs stimulated by conditioned media from macrophages stimulated by LPS. Control group represents human MSCs stimulated by unstimulated macrophage conditioned media. Expansion media group represents human MSCs without stimulation cultured in expansion media. The osteogenic group represents human MSCs without stimulation cultured in osteogenic media. Direct: LPS group represents human MSCs stimulated by LPS. Expansion media group represents human MSCs without stimulation cultured in expansion media. The osteogenic group represents human MSCs without stimulation cultured in osteogenic media*

The osteogenic effects were analysed through ALP activity, an early osteogenic marker. Human MSC were stimulated with the different groups directly or indirectly. Direct addition is defined as the direct addition of different concentrations of all the groups to human MSCs. For indirect addition the supernatant of macrophages, stimulated with all the groups in different concentrations, was added to human MSCs. The ALP activity of the groups can be seen in Figure 18. The activity was normalized to the ALP activity of human MSC cultured with only osteogenic media. For indirect addition, the control group represents the addition of unstimulated macrophage supernatant and the LPS group represents the addition of macrophage supernatant stimulated by LPS. For direct addition, the control group represents human MSCs cultured with only expansion media and the LPS group represents the direct addition of LPS to the human MSCs.

No significant effect of liposomes can be seen in the ALP activity. All the groups are similar to the osteogenic media in the indirect set-up. The condition media from macrophages seems to have a slight positive effect on the ALP activity of human MSCs. The condition media from macrophages with released liposomes from the coating seem to exert a slightly higher effect on ALP activity than the condition media from macrophages with liposomes added directly macrophages. The lower concentration of liposome present might be a more suitable concentration for increasing ALP activity. Another explanation could be the cytokine production induced by the GelMa hydrogel has an effect on the ALP activity.

When the liposomes are added directly to human MSCs, they seem to inhibit ALP activity in the higher concentrations. Furthermore can be seen that the direct stimulation of human MSCs with released liposomes from the coating seems to inhibit ALP activity. The effect that the released liposomes exert is mediated through macrophages, as can be seen by the ineffectiveness of adding the released liposomes directly to human MSCs.

## 4.2 Osteo-immunomodulatory effect of PAMPs encapsulated in LNPs

### 4.2.1 LNP characterization

Empty LNP and LNPs encapsulating CpG C and Poly(I:C) were synthesised using the ethanol dilution method composed of DPPC/DODAP/CH/DPPG in molar ratio of 38/25/30/7. The formulations used were established in precious research[60]. The characterization of the LNPs can be seen in Table 6. All LNPs had a mean particle size ranging from 150nm to 300 nm with a high monodisperse size distribution. All LNPs used had a similar negative zeta-potential.

| | *Empty – LNP* | *CpG C – LNP* | *Poly(I:C) - LNP* |
|---|---|---|---|
| Mean particle size (nm) | 198 ± 29 | 259 ± 5 | 247 ± 29 |
| PDI | 0.06 ± 0.03 | 0.15 ± 0.05 | 0.19 ± 0.04 |
| Zeta-potential (mV) | -36 ± 2 | -36 ± 2 | -40 ± 2 |

*Table 6 - LNP characterization created with the ethanol dilution method. The standard deviation was measured based on the average of the samples created.*

### 4.2.2 Osteo-immunomodulatory effect of LNPs *in vitro*

The immunomodulatory effects of empty LNP, LNPs encapsulating the PAMPs and free form of PAMPs was analysed by measuring cytokine release of human macrophages after stimulation for 24 hours. The control group consists of macrophages without stimulation and the LPS group serves as a positive control group. The cytokine production can be seen in Figure 19.

CpG C and Poly (I:C) did not increase TNF-a production in their free form in both donors, their TNF-a production is similar to the TNF-a production of the control group. When encapsulated in LNPs both CpG C and Poly (I:C) elevate the production of TNF-a in a dose dependant manner. The elevated level of TNF-a is similar in the empty LNP group. There is almost no difference between the TNF-a production between the empty-LNP group and the encapsulated PAMPs groups. This indicates that TNF-a production is mainly increased due to the intrinsic effects of the LNPs, not the intracellular uptake of CpG C or Poly (I:C) into the cells. The production of IL-6 is similar to the control group for CpG C and Poly (I:C) in their free form. The PAMPs encapsulated in LNPs increase the IL-6 production in a dose dependant manner. Empty-LNP also increase IL-6 production, however no dose dependence was observed. For both CpG C and Poly (I:C) encapsulated in the LNP there is a higher production of IL-6 compared to the empty-LNP group in high concentrations, there seems to be an additive effect if the PAMPs are encapsulated. This could indicate that CpG C and Poly (I:C) encapsulated in LNPs can increase IL-6 production because of improved intracellular uptake of CpG and Poly(I:C). The production of IL-10 had a higher donor variation compared to TNF-a and IL-6. For donor A free form PAMPs, empty-LNP and PAMPs encapsulated had a similar effect on IL-10 production. For donor B the free form PAMPs did not effect the IL-10 production, while the empty-LNP and PAMP loaded LNPs increased IL-10 production. The effect for CpG C loaded LNP and empty LNP was similar, indicating that the intrinsic immunomodulatory effect of LNPs is the reason for increased IL-10 production. A slight increase in IL-10 production could be seen in high concentration encapsulated Poly(I:C) compared to empty LNPs. Suggesting that encapsulation of Poly (I:C) slightly increases IL-10 production due to intracellular delivery of the PAMP.

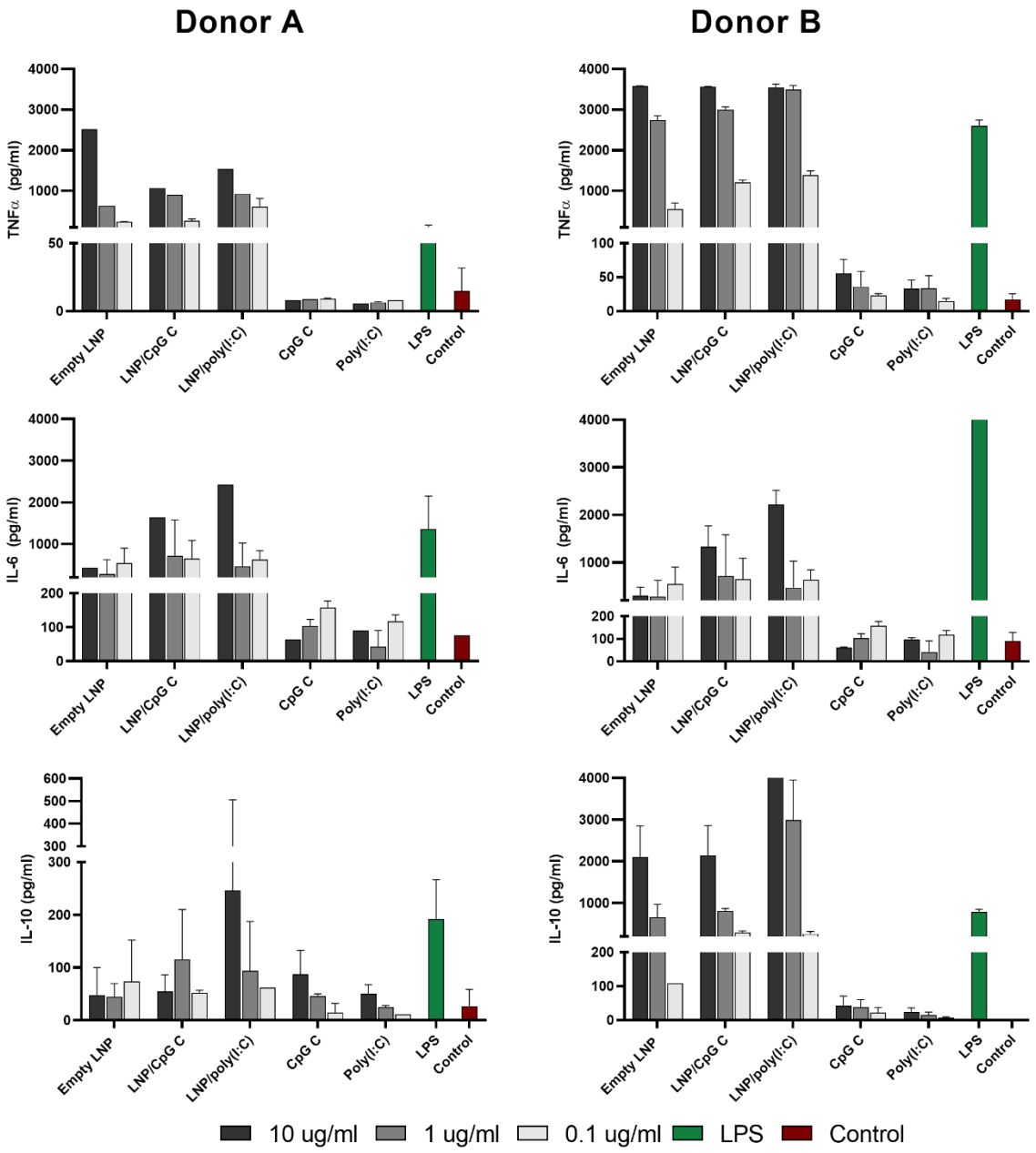


*Figure 19 - Cytokine production (TNF-a, IL-6 and IL-10) of human macrophages (2 donors) after 24 hours of stimulation with different concentrations of Empty-LNP, CpG C-LNP, Poly(I:C)-LNP, free form CpG C and free form Poly(I:C). The control group represents the cytokine production without stimulation. The LPS group represents the cytokine production after stimulation with LPS.*

The osteogenic effects were analysed through the early osteogenic marker ALP activity. The ALP activity of the groups can be seen in Figure 20. The ALP activity was measured at two different time points, day 4 and day 10 (Appendix B). The intracellular ALP activity is know to variate when different stimulations are applied to human MSCs[86,87]. The ALP activity rises in early stages of osteogenic differentiation, peaks somewhere in the first 12 days and returns to base level. At day 10 the ALP activity seemed to be almost similar for every group, it was thought that the peak in ALP activity had been earlier. Therefore was the ALP activity of day 4 used for analysis.

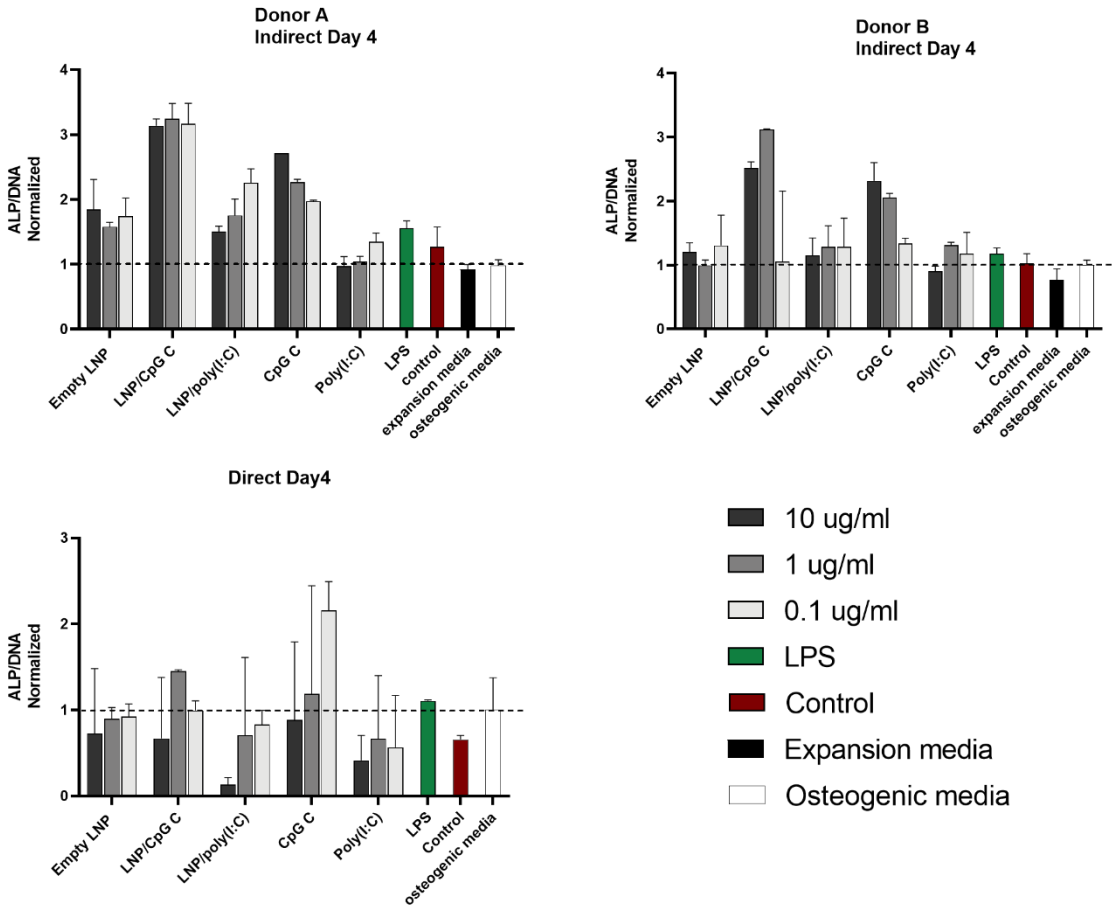


*Figure 20 - ALP activity of human MSCs at time point day 4 after indirect (Macrophage conditioned media from Donor A and B) and direct stimulation with different concentrations of Empty-LNP, CpG C-LNP, Poly(I:C)-LNP, free form CpG C and free form Poly(I:C). Indirect: LPS group represents human MSCs stimulated by conditioned media from macrophages stimulated by LPS. Control group represents human MSCs stimulated by unstimulated macrophage conditioned media. Expansion media group represents human MSCs without stimulation cultured in expansion media. The osteogenic group represents human MSCs without stimulation cultured in osteogenic media. Direct: LPS group represents human MSCs stimulated by LPS. Expansion media group represents human MSCs without stimulation cultured in expansion media. The osteogenic group represents human MSCs without stimulation cultured in osteogenic media*

The empty-LNP did not seem to have an intrinsic effect on the ALP activity, both direct and indirect addition showed no significant increase. Free form Poly(I:C) did not lead to an increase in ALP activity compared to human MSCs cultured in osteogenic media in the direct assay or in the indirect assay. The encapsulated form of Poly(I:C) showed in donor A a slight increase in ALP activity, most abundant at lower concentrations. There is no clear measurement that concludes that encapsulation of Poly(I:C) increases the ALP activity of the PRR ligand. Direct addition of encapsulated Poly(I:C) and free form Poly(I:C) to human MSCs showed a lower ALP activity than via indirect addition. This indicates that the slight effect which Poly(I:C) imposes on increased ALP activity is mediated through macrophages. This suggests that both free form and encapsulated Poly(I:C) can be taken up by macrophages. ALP activity of Human MSCs does not seem to be effected directly by encapsulated or free form Poly(I:C). It is uncertain whether LNPs encapsulating Poly (I:C) and free form are not taken up by human MSCs, or if they are taken up but do not exert any effect on ALP activity.

CpG C in its free form increased ALP activity in a dose dependent manner in the indirect assay for both donors. The encapsulated CpG C showed an even higher induction of ALP activity in the indirect

assay. The encapsulation of CpG C in LNPs increased the ALP activity compared to the free form. This indicates that encapsulation of CpG C in LNPs can induce an increased biological activation due to higher transfection into macrophages. The direct assay shows that the free form CpG C can assert an effect on ALP activity, increasing ALP activity at a low concentration. When CpG C is encapsulated in a LNP there seems to be no direct effect on ALP activity. This indicates that the effect of LNP encapsulating CpG C is mediated through macrophage uptake. Free form CpG C seems to mediates its effect through both MSCs and macrophages.

### 4.2.3 *In vivo* rabbit study

The retrieved constructs from the rabbit study were processed and stained. Following, images were taken with the microscope. All groups showed signs of scaffold degradation. Additionally, bone formation could be seen in various degrees for all the groups (Figure 21). Osteoclasts, osteoblasts and osteocytes could be identified inside all the scaffolds. Fat tissue was present in areas where bone tissue could be found at a varying degree. The remainder of BCP pores were filled with fibrous tissue.

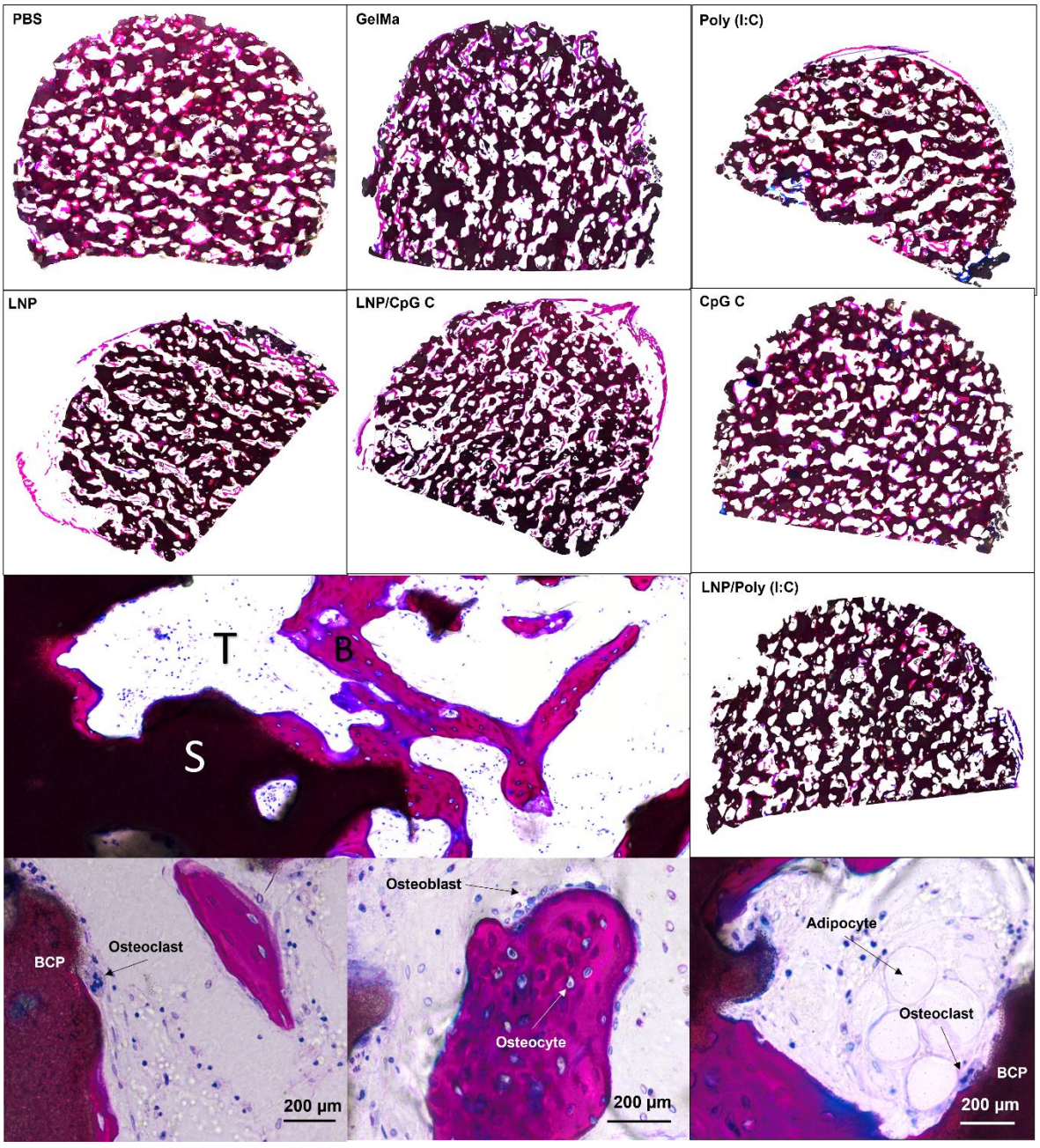


*Figure 21 – Stained cross-sections of BCP scaffold from PBS, GelMA, Poly(I:C), CpG C, LNP, LNP/CpG C, LNP/Poly(I:C) groups. Zoomed in section of scaffold with (S):scaffold, (B): bone tissue and (T): fibrous tissue. Bone cells found within the different groups, including osteoclasts, osteoblasts, osteocytes and adipocytes (BCP: BCP scaffold).*

The bone area % quantified showed a large variation between different donors for each group (Figure 22). The GelMa group, which was designed as a control, showed a similar bone area % as the PBS group, which was designed as a negative control (appendix C). No considerable difference could be seen in bone area % between the groups. Only CpG C seems to have a slight effect on the bone area %. 5 out of 8 samples from the free form and 5 out of 6 samples from the encapsulated form did increase the bone are % compared to the GelMa control in the same rabbit. The average bone area % for the encapsulated CpG C seems slightly higher compared to the other groups. Nevertheless this effect is to low to conclude a definite effect for CpG C encapsulated or in its free form.

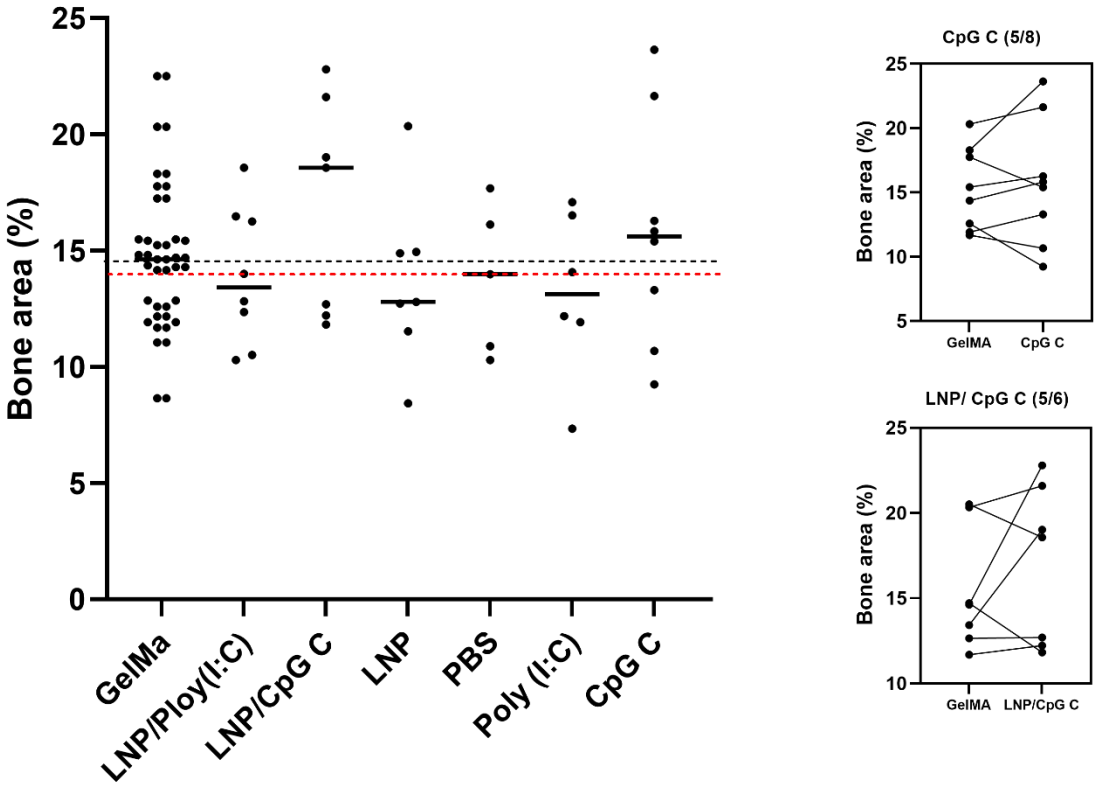


*Figure 22 – Bone area % of all the different groups (left). Black dotted line represents the average bone area % for the GelMa group. Red dotted line represents the average bone area % for the PBS group. Individual data points for CpG C and LNP/CpG C (right). The connected dots represent the bone area % in the same rabbit. The number above the graph represents for how many rabbits the bone area % increased compared to the GelMa group.*

The rabbit model used in this study does not show any definitive difference between the different groups. The influence of BMP-2 seems to be decisive on the extent of bone formation. The concentration used of LNPs and PAMPs could be too low to influence the outcome of the measurement. Furthermore is the intrinsic ability of GelMa to inducing a inflammatory response obscuring the precise effect of LNPs and PAMPs. A cleaner rabbit model without BMP-2 or less BMP-2 and a higher concentration of PAMPs might be preferable to elucidate the *in vivo* effects of the PAMPs. Additionally, the use of GelMa hydrogel should be accessed. Different hydrogels, which do not induce a inflammatory response, might be used. Another option is the use of GelMa with a lower endotoxin count, which has been shown to potentially influence to inflammatory response[85].

# 5. Conclusion

In this research the osteo-immunomodulatory influence of lipid carriers has been the focus. The first part of the research focussed on the intrinsic immunomodulatory effects of cationic and anionic liposomes and their osteogenic capacities. The anionic liposome formulation had been established in previous research[60]. The cationic liposomes were successfully synthesised with different molar fractions of DOTAP. A favourable fraction of DOTAP was found, which produced the desired positive charge at limited cost of the lipid used. Furthermore was there no sign of influential cytotoxicity from the liposomes during the experiments in this research, based on DNA content and cell activity.

Both cationic and anionic liposome showed an increase in cytokine production in murine and human macrophages compared to unstimulated controls, indicating the induction of an inflammatory response. Furthermore, a dose dependant NF-κB activation could be seen after stimulation with charged liposomes. However, no significant increase in ALP activity of human MSCs during direct or indirect stimulation could be seen. The cytokines produced by macrophages seems to positively influence ALP activity. It is impossible to say which signals cause this effect on human MSCs from this research. In this study only a few cytokines released have been measured, other signals might be present in the condition media added to the human MSCs. The pathway through which the inflammatory response is initiated might be of importance for ALP activity. A similar NF-κB activation could be seen for the charged liposomes compared to LPS. It has been shown that LPS is able to induce ALP activity in hMSC[49]. Therefore is it important to elucidate the precise pathway through which NF-κB is activated when macrophages are stimulated with charged liposomes. Different distinctive cellular responses can be activated despite shared NF-κB activation[88]. Differences in signalling pathways might elucidate the mechanism through which ALP activity is influenced and which signals play an important role. The applicability of liposomes in a hydrogel coating shows promise. However, the exact influence of the released liposomes compared to degraded hydrogel needs to be further investigated. In conclusion, the charged liposomes are able to induce an inflammatory reaction in macrophages. However, this does not seem to directly translate into a higher ALP activity in human MSCs.

The second part of this research focusses on the osteo-immunomodulatory effects of CpG C and Poly(I:C) encapsulated in LNPs. The LNP formulations were established in previous research[60]. CpG C and Poly (I:C) were successfully encapsulated in LNPs. The encapsulation of the PAMPs showed a higher cytokine production in macrophages compared to their free form. The increased inflammatory response is partially caused by the intrinsic immunomodulatory effect the LNPs themselves. TNF-a production seems to be increased mainly by the intrinsic immunomodulatory effect of LNPs. IL-6 and IL-10 production were both increased after stimulation by PAMPs encapsulated in LNPs compared to empty LNPs. Indicating an additive effect of effective transfection of CpG C and Poly(I:C) in macrophages. The ALP activity in human MSCs showed that CpG C was effective in increasing the ALP activity. This effect could be increased due to encapsulation of CpG C in the LNP. Poly(I:C) free form and encapsulated, as well as empty-LNP, showed a minimal influence on ALP activity, no clear increase could be seen. The effect on ALP activity was mediated through signals excreted by macrophages, as direct addition to human MSCs showed no increase in ALP activity. The *in vivo* rabbit study gave no clear indication for increased bone formation of the PAMPs, either free form or encapsulated in LNPs. A cleaner rabbit model might be more suitable to elucidate the *in vivo* effects. It is important for the future to expand the knowledge on the precise pathways which are involved in inducing the inflammatory reaction. Empty-LNPs seem to induce inflammation

relatively similar to encapsulated CpG C, however this is not translated towards inducing a similar ALP activity. Distinctive cellular responses can seen through activation of different TLRs[88]. It would interesting to see which precise cellular pathways are activated by empty-LNPs compared to CpG C encapsulating LNPs. Elucidating the precise mechanisms involved in the inflammation could clarify which type of inflammation is beneficial for ALP activity and therefore osteogenic capacity. In conclusion, LNPs are able to induce an inflammatory reaction in macrophages due to their intrinsic immunomodulatory effects. Encapsulation of CpG C and Poly (I:C) in LNPs has an additive effect on the inflammatory reaction. The inflammatory response is not directly translated into a higher ALP activity in human MSCs. CpG C seems most promising to induce osteogenic activity in human MSCs. The osteogenic activity could be increased by encapsulation of CpG C in LNPs.

More research is required for a conclusive result indicating the osteoinductive capacities of lipid carriers and PAMPs. Late osteogenic markers should be investigated for their expression upon stimulation of the lipid carriers and the PAMPs used in this study. Furthermore could the molecular pathways behind the inflammatory response of lipid carriers could give insight into the type of inflammation which is beneficial for osteogenesis.

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# Appendix

## Appendix A - DOTAP molar fraction effects liposome size and zeta-potential

| *DPPC/DOTAP/CH* | *34/35/31* | *61/8/31* |
|---|---|---|
| Mean particle size (nm) | 165 ± 4 | 179 ± 3 |
| PDI | 0.12 ± 0.03 | 0.15 ± 0.02 |
| Zeta-potential (mV) | 16 ± 1 | 10 ± 1 |

*Table C1- DOTAP molar fraction effect on liposome size and zeta-potential*

## Appendix B – Cytokine production and ALP activity PAMPs encapsulated in LNPs.

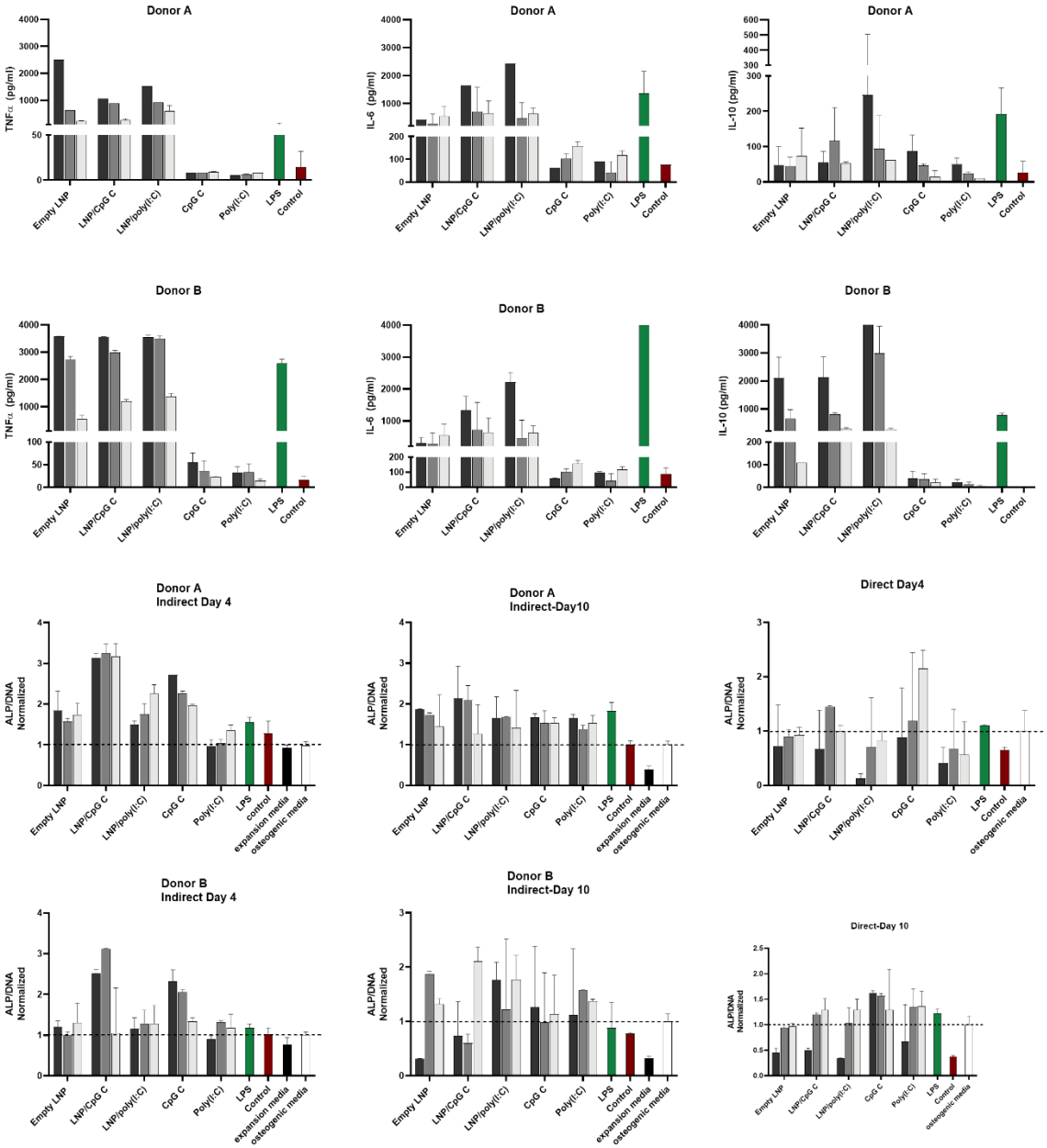


*Figure B1 - Cytokine production and ALP activity PAMPs encapsulated in LNPs*

## Appendix C – *in vivo* rabbit study

| **Group** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| GelMa | R1-1 | R3-2 | R4-2 | R6-3 | R7-2 | R8-2 | R9-3 | R10-1 | 11-3 | 12-3 |
| | R13-3 | R14-1 | R15-2 | R16-1 | R17-2 | R18-3 | R19-3 | R20-3 | 21-1 | 22-3 |
| | R23-1 | | | | | | | | | |
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
| LNP | R1-3 | R4-1 | R8-1 | R11-2 | R13-2 | R18-1 | R22-1 | | | |
| LNP/Poly(I:C) | R1-2 | R6-1 | R10-2 | R13-1 | R16-2 | R20-2 | R22-2 | R23-3 | | |
| LNP/CpG C | R10-3 | **R12-2** | R14-2 | R16-3 | R17-1 | R19-1 | R23-2 | | | |
| PBS | R3-3 | R4-3 | R9-2 | R15-1 | R18-2 | R20-1 | | | | |
| Poly (I:C) | R7-1 | R8-3 | R9-1 | R14-3 | R15-3 | R21-2 | | | | |
| CpG C | R3-1 | R6-2 | R7-3 | R11-1 | R12-1 | R17-3 | R19-2 | R21-3 | | |

*Table C1 – Division of different rabbits and intramuscular implant location over the test groups. R=rabbit number, -(1/2/3)=intramuscular implant location*

| | Bone area % | | | Bone area % | | | Bone area % |
|---|---|---|---|---|---|---|---|
| R1-1 | 14.80847 | | R11-1 | 23.63341 | | R18-1 | 14.94364 |
| R1-2 | 12.82345 | | R11-2 | 14.88693 | | R18-2 | 16.12183 |
| R1-3 | 11.53176 | | R11-3 | 18.29674 | | R18-3 | 12.85204 |
| R3-1 | 15.82326 | | R12-1 | 21.64855 | | R19-1 | 19.02113 |
| R3-2 | 14.36688 | | R12-2 | 20.05966 | | R19-2 | 16.27618 |
| R4-1 | 20.34641 | | R12-3 | 20.33134 | | R19-3 | 15.41851 |
| R4-2 | 14.15809 | | R13-1 | 18.55941 | | R20-1 | 10.28247 |
| R4-3 | 10.88405 | | R13-2 | 12.7962 | | R20-2 | 10.50369 |
| R6-1 | 16.2541 | | R13-3 | 8.646665 | | R20-3 | 12.16563 |
| R6-1 | 15.40114 | | R14-1 | 22.50916 | | R21-1 | 12.5929 |
| R6-3 | 17.75873 | | R14-2 | 18.56458 | | R21-2 | 7.336147 |
| R7-1 | 11.92353 | | R14-3 | 14.06396 | | R21-3 | 9.245284 |
| R7-2 | 11.9212 | | R15-1 | 13.98983 | | R22-1 | 12.71739 |
| R7-3 | 13.30591 | | R15-2 | 17.238 | | R22-2 | 10.28367 |
| R8-1 | 8.431977 | | R15-3 | 12.18597 | | R22-3 | 15.23528 |
| R8-2 | 11.05193 | | R16-1 | 14.29512 | | R23-1 | 14.70165 |
| R8-3 | 16.51254 | | R16-2 | 16.47037 | | R23-2 | 11.81552 |
| R9-1 | 17.08065 | | R16-3 | 12.69502 | | R23-3 | 12.35279 |
| R9-2 | 17.67325 | | R17-1 | 12.21321 | | | |
| R9-3 | 15.47962 | | R17-2 | 11.68542 | | | |
| R10-1 | 14.62061 | | R17-3 | 10.68006 | | | |
| R10-2 | 14.00457 | | | | | | |
| R10-3 | 20.79177 | | | | | | |

*Table C2 – Bone area % of each sample.*

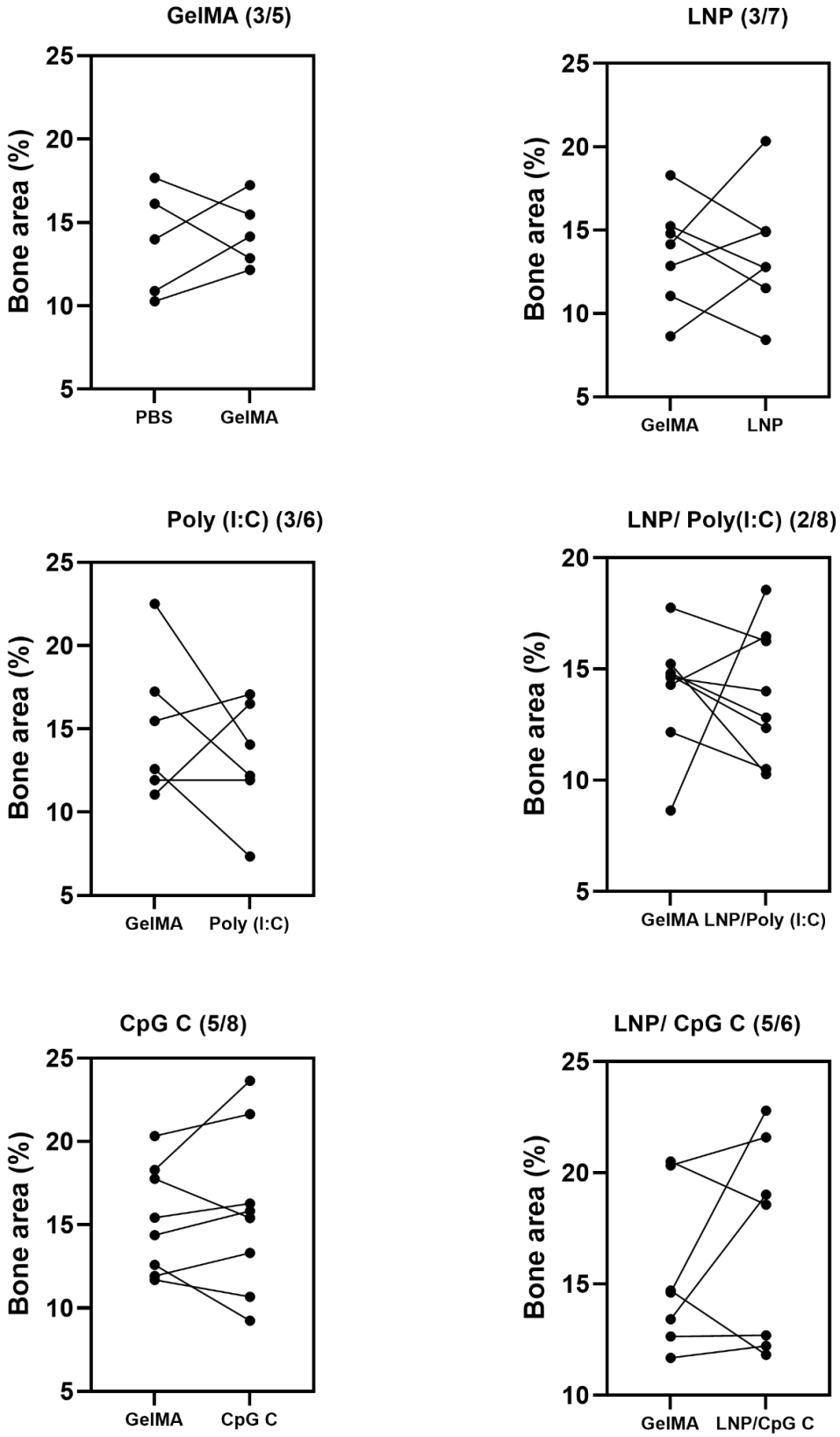


*Figure C1 - Individual data points for each group. The connected dots represent the bone area % in the same rabbit. The number above the graph represents for how many rabbits the bone area % increased compared to the GelMa group.*